The Protein Protocols Handbook

Third Edition

Edited by

John M. Walker

Humana Press

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University of Hertfordshire, Hatfield, UK

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Preface

Since the second edition of this book was published in 2002 there have, of course, been continual methodological developments in the field of protein chemistry. Consequently, for this third edition, I introduced 57 chapters/protocols not present in the second edition, significantly updated a number of chapters remaining from the second edition and increased the overall length of the book from 164 to 208 chapters. The new chapters are generally spread across the various sections of the book, but in particular we have expanded the section on post-translational modifications to reflect the increasing importance of these modifications in understanding of protein function.

As in the earlier editions, *The Protein Protocols Handbook* aims to provide a cross-section of analytical techniques commonly used to study proteins and peptides, thus providing a benchtop manual and guide for both those who are new to the protein chemistry laboratory and for more established workers who wish to use a technique for the first time.

All chapters are written in the same format as that used in the *Methods in Molecular Biology* series. Each chapter opens with a description of the basic theory behind the method being described. The Materials section lists all the chemicals, reagents, buffers, and other materials necessary for carrying out the protocol. Since the principal goal of the book is to provide experimentalists with a full account of the practical steps necessary for carrying out each protocol successfully, the Methods section contains detailed step-by-step descriptions for every protocol that should result in the successful execution of each method. The Notes section complements the Methods material by indicating how best to deal with any problem or difficulty that may arise when using a given technique, by providing useful hints and tips, and by explaining how to go about making appropriate modifications or alterations to the protocol.

John M. Walker

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PART I

QUANTITATION OF PROTEINS

1

Protein Determination by UV Absorption

Alastair Aitken and Michèle P. Learmonth

1. Introduction

1.1. Near UV Absorbance (280 nm)

Quantitation of the amount of protein in a solution is possible in a simple spectrometer. Absorption of radiation in the near UV by proteins depends on the Tyr and Trp content (and to a very small extent on the amount of Phe and disulfide bonds). Therefore the A_{280} varies greatly between different proteins (for a 1 mg/mL solution, from 0 up to 4 [for some tyrosine-rich wool proteins], although most values are in the range 0.5–1.5 *[1]*). The advantages of this method are that it is simple, and the sample is recoverable. The method has some disadvantages, including interference from other chromophores, and the specific absorption value for a given protein must be determined. The extinction of nucleic acid in the 280-nm region may be as much as 10 times that of protein at their same wavelength, and hence, a few percent of nucleic acid can greatly influence the absorption.

1.2. Far UV Absorbance

The peptide bond absorbs strongly in the far UV with a maximum at about 190 nm. This very strong absorption of proteins at these wavelengths has been used in protein determination. Because of the difficulties caused by absorption by oxygen and the low output of conventional spectrophotometers at this wavelength, measurements are more conveniently made at 205 nm, where the absorbance is about half that at 190 nm. Most proteins have extinction coefficients at 205 nm for a 1 mg/mL solution of 30–35 and between 20 and 24 at 210 nm *(2)*.

Various side chains, including those of Trp, Phe, Tyr, His, Cys, Met, and Arg (in that descending order), make contributions to the A_{205} *(3)*.

The advantages of this method include simplicity and sensitivity. As in the method outlined in **Subheading 3.1.** the sample is recoverable and in addition there

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is little variation in response between different proteins, permitting near-absolute determination of protein. Disadvantages of this method include the necessity for accurate calibration of the spectrophotometer in the far UV. Many buffers and other components, such as heme or pyridoxal groups, absorb strongly in this region.

2. Materials

1. 0.1 *M* K_2SO_4 (pH 7.0).
2. 5 m*M* potassium phosphate buffer, pH 7.0.
3. Nonionic detergent (0.01% Brij 35)
4. Guanidinium-HCl.
5. 0.2-µm Millipore (Watford, UK) filter.
6. UV-visible spectrometer: The hydrogen lamp should be selected for maximum intensity at the particular wavelength.
7. Cuvets, quartz, for <215 nm.

3. Methods

3.1. Estimation of Protein by Near UV Absorbance (280 nm)

1. A reliable spectrophotometer is necessary. The protein solution must be diluted in the buffer to a concentration that is well within the accurate range of the instrument (*see* **Notes 1** and **2**).
2. The protein solution to be measured can be in a wide range of buffers, so it is usually no problem to find one that is appropriate for the protein which may already be in a particular buffer required for a purification step or assay for enzyme activity, for example (*see* **Notes 3** and **4**).
3. Measure the absorbance of the protein solution at 280 nm, using quartz cuvets or cuvets that are known to be transparent to this wavelength, filled with a volume of solution sufficient to cover the aperture through which the light beam passes.
4. The value obtained will depend on the path length of the cuvet. If not 1 cm, it must be adjusted by the appropriate factor. The Beer-Lambert law states that:

   $$A\,(\text{absorbance}) = \varepsilon\, c\, 1 \qquad (1)$$

   where ε = extinction coefficient, *c* = concentration in mol/L and *l* = optical path length in cm. Therefore, if ε is known, measurement of *A* gives the concentration directly, ε is normally quoted for a 1-cm path length.
5. The actual value of UV absorbance for a given protein must be determined by some absolute method, e.g., calculated from the amino acid composition, which can be determined by amino acid analysis ***(4)***. The UV absorbance for a protein is then calculated according to the following formula:

   $$A_{280}\,(1\,\text{mg/mL}) = (5690n_{\text{w}} + 1280n_{y} + 120n_{c}) \,/\, M \qquad (2)$$

   where n_{w}, n_{y}, and n_{c} are the numbers of Trp, Tyr, and Cys residues in the polypeptide of mass *M* and 5690, 1280 and 120 are the respective extinction coefficients for these residues (*see* **Note 5**).

3.2. Estimation of Protein by Far UV Absorbance

1. The protein solution is diluted with a sodium chloride solution (0.9% w/v) until the absorbance at 215 nm is <1.5 (*see* **Notes 1** and **6**).
2. Alternatively, dilute the sample in another non-UV-absorbing buffer such as 0.1 *M* K_2SO_4, containing 5 m*M* potassium phosphate buffer adjusted to pH 7.0 (*see* **Note 6**).
3. Measure the absorbances at the appropriate wavelengths (either A_{280} and A_{205}, or A_{225} and A_{215}, depending on the formula to be applied), using a spectrometer fitted with a hydrogen lamp that is accurate at these wavelengths, using quartz cuvets filled with a volume of solution sufficient to cover the aperture through which the light beam passes (details in **Subheading 3.1**).
4. The A_{205} for a 1 mg/mL solution of protein ($A_{205}{}^{1\,\text{mg/mL}}$) can be calculated within ±2%, according to the empirical formula proposed by Scopes ***(2)*** (*see* **Notes 7–10**):

$$A_{205}{}^{1\,\text{mg/mL}} = 27 + 120\,(A_{280} / A_{205}) \qquad (3)$$

5. Alternatively, measurements may be made at longer wavelengths ***(5)***:

$$\text{Protein concentration}\,(\mu\text{g/mL}) = 144\,(A_{215} - A_{225}) \qquad (4)$$

The extinction at 225 nm is subtracted from that at 215 nm; the difference multiplied by 144 gives the protein concentration in the sample in µg/mL. With a particular protein under specific conditions accurate measurements of concentration to within 5 µg/L are possible.

4. Notes

1. It is best to measure absorbances in the range 0.05–1.0 (between 10 and 90% of the incident radiation). At around 0.3 absorbance (50% absorption), the accuracy is greatest.
2. Bovine serum albumin is frequently used as a protein standard; 1 mg/mL has an A_{280} of 0.66.
3. If the solution is turbid, the apparent A_{280} will be increased by light scattering. Filtration (through a 0.2-µm Millipore filter) or clarification of the solution by centrifugation can be carried out. For turbid solutions, a convenient approximate correction can be applied by subtracting the A_{310} (proteins do not normally absorb at this wavelength unless they contain particular chromophores) from the A_{280}.
4. At low concentrations, protein can be lost from solution by adsorption on the cuvet; the high ionic strength helps to prevent this. Inclusion of a nonionic detergent (0.01% Brij 35) in the buffer may also help to prevent these losses.
5. The presence of non-protein chromophores (e.g., heme, pyridoxal) can increase A_{280}. If nucleic acids are present (which absorb strongly at 260 nm), the following formula can be applied. This gives an accurate estimate of the protein content by removing the contribution to absorbance by nucleotides at 280 nm, by measuring the A_{260} which is largely owing to the latter ***(6)***.

$$\text{Protein (mg/mL)} = 1.55\,A_{280} - 0.76\,A_{260} \quad (5)$$

Other formulae (using similar principles of absorbance differences) employed to determine protein in the possible presence of nucleic acids are the following *(7,8)*:

$$\text{Protein (mg/mL)} = (A_{235} - A_{280})\,/\,2.51 \quad (6)$$

$$\text{Protein (mg/mL)} = 0.183\,A_{230} - 0.075.8\,A_{260} \quad (7)$$

6. Protein solutions obey Beer-Lambert's Law at 215 nm provided the absorbance is <2.0.
7. Strictly speaking, this value applies to the protein in 6 *M* guanidinium-HCl, but the value in buffer is generally within 10% of this value, and the relative absorbances in guanidinium-HCl and buffer can be easily determined by parallel dilutions from a stock solution.
8. Sodium chloride, ammonium sulfate, borate, phosphate, and Tris do not interfere, whereas 0.1 *M* acetate, succinate, citrate, phthalate, and barbiturate show high absorption at 215 nm.
9. The absorption of proteins in the range 215–225 nm is practically independent of pH between pH values 4–8.
10. The specific extinction coefficient of a number of proteins and peptides at 205 nm and 210 nm ***(3)*** has been determined. The average extinction coefficient for a 1 mg/mL solution of 40 serum proteins at 210 nm is 20.5 ± 0.14. At this wavelength, a protein concentration of 2 µg/mL gives $A = 0.04$ ***(5)***.

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2

The Lowry Method for Protein Quantitation

Jakob H. Waterborg

1. Introduction

The most accurate method of determining protein concentration is probably acid hydrolysis followed by amino acid analysis. Most other methods are sensitive to the amino acid composition of the protein, and absolute concentrations cannot be obtained ***(1)***. The procedure of Lowry et al. ***(2)*** is no exception, but its sensitivity is moderately constant from protein to protein, and it has been so widely used that Lowry protein estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances in which protein mixtures or crude extracts are involved.

The method is based on both the Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu^+, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 mg of protein/mL, and is best used on solutions with concentrations in the range 0.01–1.0 mg/mL of protein.

2. Materials

1. Complex-forming reagent: Prepare immediately before use by mixing the following stock solutions in the proportion 100:1:1 (by vol), respectively:
   Solution A: 2% (w/v) Na_2CO_3 in distilled water.
   Solution B: 1% (w/v) $CuSO_4$ $5H_2O$ in distilled water.
   Solution C: 2% (w/v) sodium potassium tartrate in distilled water.
2. 2 *N* NaOH.
3. Folin reagent (commercially available): Use at 1 *N* concentration.

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4. Standards: Use a stock solution of standard protein (e.g., bovine serum albumin fraction V) containing 2 mg/mL protein in distilled water, stored frozen at −20°C. Prepare standards by diluting the stock solution with distilled water as follows:

Stock solution (µL)	0	2.5	5	12.5	25	50	125	250	500
Water (µL)	500	498	495	488	475	450	375	250	0
Protein conc. (µg/mL)	0	10	20	50	100	200	500	1000	2000

3. Method

1. To 0.1 mL of sample or standard (*see* **Notes 1–4**), add 0.1 mL of 2 *N* NaOH. Hydrolyze at 100°C for 10 min in a heating block or boiling water bath.
2. Cool the hydrolysate to room temperature and add 1 mL of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 min (*see* **Notes 5** and **6**).
3. Add 0.1 mL of Folin reagent, using a vortex mixer, and let the mixture stand at room temperature for 30–60 min (do not exceed 60 min) (*see* **Note 7**).
4. Read the absorbance at 750 nm if the protein concentration was below 500 µg/mL or at 550 nm if the protein concentration was between 100 and 2000 µg/mL.
5. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations (*see* **Notes 8–13**).

4. Notes

1. If the sample is available as a precipitate, then dissolve the precipitate in 2 *N* NaOH and hydrolyze as described in **Subheading 3., step 1**. Carry 0.2-mL aliquots of the hydrolyzate forward to **Subheading 3., step 2**.
2. Whole cells or other complex samples may need pretreatment, as described for the Burton assay for DNA ***(3)***. For example, the perchloroacetic acid (PCA)/ethanol precipitate from extraction I may be used directly for the Lowry assay, or the pellets remaining after the PCA hydrolysis step (**Subheading 3., step 3** of the Burton assay) may be used for Lowry. In this latter case, both DNA and protein concentration may be obtained from the same sample.
3. Peterson ***(4)*** has described a precipitation step that allows the separation of the protein sample from interfering substances and also consequently concentrates the protein sample, allowing the determination of proteins in dilute solution. Peterson's precipitation step is as follows:
    a. Add 0.1 mL of 0.15% deoxycholate to 1.0 mL of protein sample.
    b. Vortex-mix, and stand at room temperature for 10 min.
    c. Add 0.1 mL of 72% trichloroacetic acid (TCA), vortex-mix, and centrifuge at 1000–3000 *g* for 30 min.
    d. Decant the supernatant and treat the pellet as described in **Note 1**.
4. Detergents such as sodium dodecyl sulfate (SDS) are often present in protein preparations, added to solubilize membranes or remove interfering

substances ***(5–7)***. Protein precipitation by TCA may require phosphotungstic acid (PTA) ***(6)*** for complete protein recovery:

- a. Add 0.2 mL of 30% (w/v) TCA and 6% (w/v) PTA to 1.0 mL of protein sample.
- b. Vortex-mix, and stand at room temperature for 20 min.
- c. Centrifuge at 2000 *g* and 4°C for 30 min.
- d. Decant the supernatant completely and treat the pellet as described in **Note 1**.

5. The reaction is very pH dependent, and it is therefore important to maintain the pH between 10 and 10.5. Therefore, take care when analyzing samples that are in strong buffer outside this range.
6. The incubation period is not critical and can vary from 10 min to several hours without affecting the final absorbance.
7. The vortex-mixing step is critical for obtaining reproducible results. The Folin reagent is reactive only for a short time under these alkaline conditions, being unstable in alkali, and great care should therefore be taken to ensure thorough mixing.
8. The assay is not linear at higher concentrations. Ensure that you are analyzing your sample on the linear portion of the calibration curve.
9. A set of standards is needed with each group of assays, preferably in duplicate. Duplicate or triplicate unknowns are recommended.
10. One disadvantage of the Lowry method is the fact that a range of substances interferes with this assay, including buffers, drugs, nucleic acids, and sugars. (The effect of some of these agents is shown in Table 1 in Chapter 3.) In many cases, the effects of these agents can be minimized by diluting them out, assuming that the protein concentration is sufficiently high to still be detected after dilution. When interfering compounds are involved, it is, of course, important to run an appropriate blank. Interference caused by detergents, sucrose, and EDTA can be eliminated by the addition of SDS ***(5)*** and a precipitation step (*see* **Note 4**).
11. Modifications to this basic assay have been reported that increase the sensitivity of the reaction. If the Folin reagent is added in two portions, vortex-mixing between each addition, a 20% increase in sensitivity is achieved ***(8)***. The addition of dithiothreitol 3 min after the addition of the Folin reagent increases the sensitivity by 50% ***(9)***.
12. The amount of color produced in this assay by any given protein (or mixture of proteins) is dependent on the amino acid composition of the protein(s) (*see* Introduction). Therefore, two different proteins, each for example at concentrations of 1 mg/mL, can give different color yields in this assay. It must be appreciated, therefore, that using bovine serum albumin (BSA) (or any other protein for that matter) as a standard gives only an approximate measure of

the protein concentration. The only time when this method gives an absolute value for protein concentration is when the protein being analyzed is also used to construct the standard curve. The most accurate way to determine the concentration of any protein solution is amino acid analysis.

13. A means of speeding up this assay using raised temperatures ***(10)*** or a microwave oven (*see* Chapter 5) has been described.

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3

The Bicinchoninic Acid (BCA) Assay for Protein Quantitation

John M. Walker

1. Introduction

The bicinchoninic acid (BCA) assay, first described by Smith et al. ***(1)*** is similar to the Lowry assay, since it also depends on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions (*see* Chapter 2). The Cu^{+} is then detected by reaction with BCA. The two assays are of similar sensitivity, but since BCA is stable under alkali conditions, this assay has the advantage that it can be carried out as a one-step process compared to the two steps needed in the Lowry assay. The reaction results in the development of an intense purple color with an absorbance maximum at 562 nm. Since the production of Cu^{+} in this assay is a function of protein concentration and incubation time, the protein content of unknown samples may be determined spectrophotometrically by comparison with known protein standards. A further advantage of the BCA assay is that it is generally more tolerant to the presence of compounds that interfere with the Lowry assay. In particular it is not affected by a range of detergents and denaturing agents such as urea and guanidinium chloride, although it is more sensitive to the presence of reducing sugars. Both a standard assay (0.1–1.0 mg protein/mL) and a microassay (0.5–10 µg protein/mL) are described.

2. Materials

2.1. Standard Assay

1. Reagent A: sodium bicinchominate (1.0 g), Na_2CO_3 (2.0 g), sodium tartrate (dihydrate) (0.16 g), NaOH (0.4 g), $NaHCO_3$ (0.95 g), made up to 100 mL. If necessary, adjust the pH to 11.25 with $NaHCO_3$ or NaOH (*see* **Note 1**).
2. Reagent B: $CuSO_4$ $5H_2O$ (0.4 g) in 10 mL of water (*see* **Note 1**).
3. Standard working reagent (SWR): Mix 100 vol of regent A with 2 vol of reagent B. The solution is apple green in color and is stable at room temperature for 1 wk.

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2.2. Microassay

1. Reagent A: Na_2CO_3 (0.8 g), NaOH (1.6 g), sodium tartrate (dihydrate) (1.6 g), made up to 100 mL with water, and adjusted to pH 11.25 with 10 *M* NaOH.
2. Reagent B: BCA (4.0 g) in 100 mL of water.
3. Reagent C: $CuSO_4$ $5H_2O$ (0.4 g) in 10 mL of water.
4. Standard working reagent (SWR): Mix 1 vol of reagent C with 25 vol of reagent B, then add 26 vol of reagent A.

3. Methods

3.1. Standard Assay

1. To a 100-µL aqueous sample containing 10–100 µg protein, add 2 mL of SWR. Incubate at 60°C for 30 min (*see* **Note 2**).
2. Cool the sample to room temperature, then measure the absorbance at 562 nm (*see* **Note 3**).
3. A calibration curve can be constructed using dilutions of a stock 1 mg/mL solution of bovine serum albumin (BSA) (*see* **Note 4**).

3.2. Microassay

1. To 1.0 mL of aqueous protein solution containing 0.5–1.0 µg of protein/mL, add 1 mL of SWR.
2. Incubate at 60°C for 1 h.
3. Cool, and read the absorbance at 562 nm.

4. Notes

1. Reagents A and B are stable indefinitely at room temperature. They may be purchased ready prepared from Pierce, Rockford, IL.
2. The sensitivity of the assay can be increased by incubating the samples longer. Alternatively, if the color is becoming too dark, heating can be stopped earlier. Take care to treat standard samples similarly.
3. Following the heating step, the color developed is stable for at least 1 h.
4. Note, that like the Lowry assay, response to the BCA assay is dependent on the amino acid composition of the protein, and therefore an absolute concentration of protein cannot be determined. The BSA standard curve can only therefore be used to compare the relative protein concentration of similar protein solutions.
5. Some reagents interfere with the BCA assay, but nothing like as many as with the Lowry assay (*see* **Table 1**). The presence of lipids gives excessively high absorbances with this assay *(2)*. Variations produced by buffers with sulfhydryl agents and detergents have been described *(3)*.
6. Since the method relies on the use of Cu^{2+}, the presence of chelating agents such as EDTA will of course severely interfere with the method. However,

Table 1
Effect of Selected Potential Interfering Compounds[a]

Sample (50 µg BSA) in the following	BCA assay (µg BSA found) Water blank corrected	BCA assay (µg BSA found) Interference blank corrected	Lowry assay (µg BSA found) Water blank corrected	Lowry assay (µg BSA found) Interference blank corrected
50 µg BSA in water (reference)	50.00	—	50.00	—
0.1 *N* HCl	50.70	50.80	44.20	43.80
0.1 *N* NaOH	49.00	49.40	50.60	50.60
0.2% Sodium azide	51.10	50.90	49.20	49.00
0.02% Sodium azide	51.10	51.00	49.50	49.60
1.0 *M* Sodium chloride	51.30	51.10	50.20	50.10
100 m*M* EDTA (4 Na)	No color		138.50	5.10
50 m*M* EDTA (4 Na)	28.00	29.40	96.70	6.80
10 m*M* EDTA (4 Na)	48.80	49.10	33.60	12.70
50 m*M* EDTA (4 Na), pH 11.25	31.50	32.80	72.30	5.00
4.0 *M* Guanidine HCl	48.30	46.90	Precipitated	
3.0 *M* Urea	51.30	50.10	53.20	45.00
1.0% Triton X-100	50.20	49.80	Precipitated	
1.0% SDS (lauryl)	49.20	48.90	Precipitated	
1.0% Lubrol	50.70	50.70	Precipitated	
1.0% Chaps	49.90	49.50	Precipitated	
1.0% Chapso	51.80	51.00	Precipitated	
1.0% Octyl glucoside	50.90	50.80	Precipitated	
40.0% Sucrose	55.40	48.70	4.90	28.90
10.0% Sucrose	52.50	50.50	42.90	41.10
1.0% Sucrose	51.30	51.20	48.40	48.10
100 m*M* Glucose	245.00	57.10	68.10	61.70
50 mM Glucose	144.00	47.70	62.70	58.40
10 m*M* Glucose	70.00	49.10	52.60	51.20
0.2 *M* Sorbitol	42.90	37.80	63.70	31.00
0.2 *M* Sorbitol, pH 11.25	40.70	36.20	68.60	26.60
1.0 *M* Glycine	No color		7.30	7.70
1.0 *M* Glycine, pH 11	50.70	48.90	32.50	27.90
0.5 *M* Tris	36.20	32.90	10.20	8.80
0.25 *M* Tris	46.60	44.00	27.90	28.10
0.1 *M* Tris	50.80	49.60	38.90	38.90
0.25 *M* Tris, pH 11.25	52.00	50.30	40.80	40.80
20.0% Ammonium sulfate	5.60	1.20	Precipitated	
10.0% Ammonium sulfate	16.00	12.00	Precipitated	
3.0% Ammonium sulfate	44.90	42.00	21.20	21.40

(continued)

Table 1
(continued)

Sample (50 μg BSA) in the following	BCA assay (μg BSA found)		Lowry assay (μg BSA found)	
	Water blank corrected	Interference blank corrected	Water blank corrected	Interference blank corrected
2.0 *M* Sodium acetate, pH 5.5	35.50	34.50	5.40	3.30
0.2 *M* Sodium acetate, pH 5.5	50.80	50.40	47.50	47.60
1.0 *M* Sodium phosphate	37.10	36.20	7.30	5.30
0.1 *M* Sodium phosphate	50.80	50.40	46.60	46.60
0.1 *M* Cesium bicarbonate	49.50	49.70	Precipitated	

[a]Reproduced from **ref**. ***1*** with permission from Academic Press Inc.

it may be possible to overcome such problems by diluting the sample as long as the protein concentration remains sufficiently high to be measurable. Similarly, dilution may be a way of coping with any agent that interferes with the assay (*see* **Table 1**). In each case it is of course necesary to run an appropriate control sample to allow for any residual color development. A modification of the assay has been described that overcomes lipid interference when measuring lipoprotein protein content ***(4)***.

7. A modification of the BCA assay, utilizing a microwave oven, has been described that allows protein determination in a matter of seconds (*see* Chapter 5).
8. A method has been described for eliminating interfering compounds such as thiols and reducing sugars in this assay. Proteins are bound to nylon membranes and exhaustively washed to remove interfering compounds; then the BCA assay is carried out on the membrane-bound protein ***(5)***.
9. A comparison of the BCA, Lowry and Bradford assays for analyzing gylcosylated and non-glycosylated proteins have been made ***(6)***. Significant differences wee observed between the assays for non-glycosylated proteins with the BCA assay giving results closest to those from amino acid analysis. Glycosylated proteins were underestimated by the Bradford the method and overestimated by the BCA and Lowry methods. The results suggest a potential interference of protein glycosylation with colorimetric assays.
10. A modification of this assay for analysis complex samples, which involves removing contaminants from the protein precipitate with 1 *M* HCl has been reported ***(7)***.

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4

The Bradford Method For Protein Quantitation

Nicholas J. Kruger

1. Introduction

A rapid and accurate method for the estimation of protein concentration is essential in various areas of biology and biochemistry. An assay originally described by Bradford (***1***) has become the preferred method for quantifying protein in many laboratories. This technique is simpler, faster, and more sensitive than the Lowry method. Moreover, when compared with the Lowry method, it is subject to less interference by common reagents and non-protein components of biological samples (*see* **Note 1**). Despite the introduction of alternative protein assays, the Bradford method remains a popular technique, with the original article ***(1)*** being cited over 3,500 times in primary research papers in 2006, thirty years after its initial publication.

The Bradford assay relies on the binding of the dye Coomassie Blue G250 to protein. Detailed studies indicate that the free dye can exist in four different ionic forms for which the pK_a values are 1.15, 1.82 and 12.4 ***(2)***. Of the three charged forms of the dye that predominate in the acidic assay reagent solution, the more cationic red and green forms have absorbance maxima at 470 nm and 650 nm, respectively. In contrast, the more anionic blue form of the dye, which binds to protein, has an absorbance maximum at 590 nm. Thus, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595 nm (*see* **Note 2**).

The dye appears to bind most strongly to arginine and lysine residues of proteins and also, to lesser extents, histidine and aromatic residues (tryptophan, tyrosine and phenylalanine) ***(3,4)***. This specificity can lead to variation in the response of the assay to different proteins, which is the main drawback of the method (*see* **Note 3**). The original Bradford assay shows large variation in

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response between different proteins *(5–7)*. Several modifications to the method have been developed to overcome this problem (*see* **Note 4**). However, these changes generally result in a less robust assay that is often more susceptible to interference by other chemicals. Consequently, the original method devised by Bradford remains the most convenient and widely used formulation. Two types of assay are described here: the standard assay which is suitable for measuring between 10 and 100 µg protein, and the microassay for detecting between 1 and 10 µg protein. Although the latter is more sensitive, it is also more prone to interference from other compounds because of the greater amount of sample relative to assay reagent in this form of the assay.

2. Materials

1. Assay reagent: dissolve 100 mg of Coomassie Blue G250 in 50 mL of 95% ethanol. The solution is then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water (*see* **Note 5**).

   The reagent should be filtered through Whatman No. 1 filter paper and then stored in an amber bottle at room temperature. It is stable for several weeks. However, during this time dye may precipitate from solution and so the stored reagent should be filtered before use.
2. Protein standard (*see* **Note 6**): bovine γ-globulin at a concentration of 1mg/mL (100 µg/mL for the microassay) in distilled water is used as a stock solution. This should be stored frozen in small aliquots at −20°C. Since the moisture content of solid protein may vary during storage, the precise concentration of protein in the standard solution should be determined from its absorbance at 280 nm. The absorbance of a 1 mg/mL solution of γ-globulin, in a 1-cm light path, is 1.35. The corresponding values for two alternative protein standards, bovine serum albumin and ovalbumin, are 0.66 and 0.75, respectively.
3. Plastic and glassware used in the assay should be absolutely clean and detergent-free. Quartz (silica) spectrophotometer cuvettes should not be used, since the dye in the assay reagent binds to this material and thereby interferes with the assay. Traces of dye bound to glassware or plastic can be removed by rinsing with methanol or detergent solution.

3. Methods

3.1. Standard Assay Method

1. Pipet between 10 and 100 µg of protein in 100 µL total volume into a test tube. If the approximate protein concentration in the sample is unknown, assay a range of dilutions (1, 1/10, 1/100, 1/1000). Prepare duplicates of each sample.
2. For the calibration curve, pipet duplicate volumes of 10, 20, 40, 60, 80 and 100 µL of 1 mg/mL γ-globulin standard solution into test tubes, and make each up to 100 µL with distilled water. Pipet 100 µL of distilled water into a further tube to provide the reagent blank.
3. Add 5 mL of the assay reagent to each tube and mix well by inversion or gentle vortexing. Avoid foaming, which will lead to poor reproducibility.

4. Measure the A_{595} of the samples and protein standards against the reagent blank between 5 and 60 minutes after mixing (*see* **Note 7** *and* **Note 8**). The assay of the standard containing 100 μg protein should give an A_{595} value of about 0.4. The standard curve is not linear and the precise absorbance varies depending on the age of the assay reagent. Consequently, it is essential to construct a calibration curve for each set of assays (*see* **Note 9**).

3.2. Microassay Method

This form of the assay is more sensitive than the standard assay method described in 3.1. Consequently, it is useful when the amount of protein is limited (*see also* **Note 10**).

1. Pipet duplicate samples containing between 1 and 10 μg in a total volume of 100 μL into 1.5-mL polyethylene microfuge tubes. If the approximate protein concentration in the sample is unknown, assay a range of dilutions (1, 1/10, 1/100, 1/1000).
2. For the calibration curve, pipet duplicate volumes of 10, 20, 40, 60, 80 and 100 μL of 100 μg/mL γ-globulin standard solution into 1.5-mL microfuge tubes, and adjust the volume to 100 μL with water. Pipet 100 μL of distilled water into an additional microfuge tube to provide the reagent blank.
3. Add 1 mL of the assay reagent to each microfuge tube and mix gently, but thoroughly.
4. Measure the absorbance of each sample between 5 and 60 minutes after addition of the protein reagent (*see* **Note 7** *and* **Note 8**). The A_{595} value of a sample containing 10 μg γ-globulin is about 0.45. Figure 1 shows the typical response of three common protein standards using the microassay method.

4. Notes

1. The Bradford assay is relatively free from interference by most commonly used biochemical reagents. However, a few chemicals may significantly alter the absorbance of the reagent blank or modify the response of proteins to the dye (**Table 1**). The materials that are most likely to cause problems in biological extracts are detergents and ampholyte ***(3,8)***. These can be removed from the sample solution by gel filtration, dialysis or precipitation of protein with calcium phosphate ***(9,10)***. Alternatively, they can be included in the reagent blank and calibration standards at the same concentration as that found in the sample. The presence of base in the assay increases absorbance by shifting the equilibrium of the free dye toward the anionic form. This may present problems when measuring protein content in concentrated basic buffers ***(3)***. Guanidine hydrochloride and sodium ascorbate compete with dye for protein, leading to underestimation of the protein content ***(3)***. Additional care should be taken when attempting to quantify protein content in cruder biological samples. For example, measurement of protein in soil extracts is complicated by the presence of a range of polyphenolic compounds in humus that also bind to Coomassie Blue G250 thereby potentially overestimating soil protein content ***(11)***.

Table 1
Effects of Common Reagents on the Bradford Assay

	Absorbance at 600 nm	
Compound	Blank	Immunoglobulin
Control	0.005	0.264
0.02% SDS	0.003	0.250
0.1% SDS	0.042*	0.059*
0.1% Triton	0.000	0.278
0.5% Triton	0.051*	0.311*
1 M 2-Mercaptoethanol	0.006	0.273
1 M Sucrose	0.008	0.261
4 M Urea	0.008	0.261
4 M NaCl	–0.015	0.207*
Glycerol	0.014	0.238*
0.1 M Hepes (pH 7.0)	0.003	0.268
0.1 M Tris (pH 7.5)	–0.008	0.261
0.1 M Citrate (pH 5.0)	0.015	0.249
10 mM EDTA	0.007	0.235*
1 M $(NH_4)_2SO_4$	0.002	0.269

Data were obtained by mixing 5 µL of water (blank) or a solution containing 10 µg bovine immunoglobulin G with 5 µL of the specified compound before adding 200 µL of assay reagent. The asterisks indicate measurements that differ from the control by more than 0.02 absorbance unit for blank values or more than 10% for the samples containing protein. Data taken from ***ref. 7***.

2. Binding of protein to Coomassie Blue G250 shifts the absorbance maximum of the blue ionic form of the dye from 590 nm to 620 nm ***(2)***. It might, therefore, appear more sensible to measure the absorbance at the higher wavelength. However, at the usual pH of the assay, an appreciable proportion of the dye is in the green form (λ_{max} = 650 nm) which interferes with absorbance measurement of the dye-protein complex at 620 nm. Measurement at 595 nm represents the best compromise between maximising the absorbance due to the dye-protein complex while minimizing to the contribution from the green form of the free dye (***2–4***; *but see also* **Note 10**).
3. Under normal assay conditions that involve comparatively high molar protein/dye ratios the dye binds almost exclusively to arginine and lysine residues ***(4)***. The dye does not bind to free arginine or lysine, nor to peptides smaller than about 3000 Da ***(3,12)***. Many peptide hormones and other important bioactive peptides fall into the latter category, and the Bradford assay is not suitable for quantifying the amounts of such compounds.
4. The assay technique described here is subject to variation in sensitivity between individual proteins (*see* **Table 2**). Several modifications have been

suggested that reduce this variability ***(5–7,13)***. Generally, these rely on increasing either the dye content or the pH of the solution. In one variation, adjusting the pH by adding NaOH to the assay reagent improves the sensitivity of the assay and greatly reduces the variation observed with different proteins ***(7)***. (This is presumably due to an increase the proportion of free dye in the blue form, the ionic species that reacts with protein.) However, the optimum pH is critically dependent on the source and concentration of the dye (*see* **Note 5**). Moreover, the modified assay is far more sensitive to interference from detergents in the sample.

Particular care should be taken when measuring the protein content of membrane fractions. The conventional assay consistently underestimates the amount of protein in membrane-rich samples. Pre-treatment of the samples with membrane-disrupting agents such as NaOH or detergents may reduce this problem, but the results should be treated with caution ***(14)***. A useful alternative is to precipitate protein from the sample using calcium phosphate and then remove contaminating lipids (and other interfering substances, *see* **Note 1**) by washing with 80% ethanol ***(9,10)***.

5. The amount of soluble dye in Coomassie Blue G250 varies considerably between sources, and suppliers' figures for dye purity are not a reliable estimate of the Coomassie Blue G250 content ***(15)***. Generally, Serva Blue G is regarded to have the greatest dye content and should be used in the modified assays discussed in **Note 4**. However, the quality of the dye is not critical for routine protein determination using the method described in this chapter. The data presented in **Figure 1** were obtained using Brilliant Blue G (C.I. 42655; Product code B0770, Sigma-Aldrich Co.) which has a dye content of approximately 90% as determined by elemental analysis.
6. Whenever possible the protein used to construct the calibration curve should be the same as that being determined. Often this is impractical and the dye-response of a sample is quantified relative to that of a "generic" protein. Bovine serum albumin is commonly used as the protein standard because it is inexpensive and readily available in a pure form. The major argument for using this protein is that it allows the results to be compared directly with those of the many previous studies that have used bovine serum albumin as a standard. However, it suffers from the disadvantage of exhibiting an unusually large dye-response in the Bradford assay and, thus, may under-estimate the protein content of a sample. Increasingly, bovine γ-globulin is being promoted as a more suitable general standard since the dye binding capacity of this protein is closer to the mean of those proteins that have been compared (**Table 2**). Because of the variation in response between different proteins, it is essential to specify the protein standard used when reporting measurements of protein amounts using the Bradford assay.

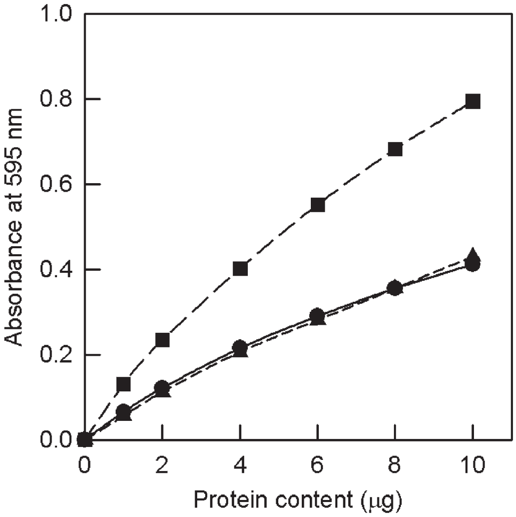

Fig. 1. Variation in the response of proteins in the Bradford assay. The extent of protein-dye complex formation was determined for bovine serum albumin (■), bovine γ-globulin (●) and chicken ovalbumin (▲) using the microassay method. Each value is the mean of four determinations. For each set of measurements the standard error was less than 5% of the mean value. The data allow comparisons to be made between estimates of protein content obtained using these protein standards.

7. The timing of these measurements is not critical, although it is better to be consistent. Sufficient time must be allowed for color development to be complete. The speed of formation of the dye-protein complex is temperature dependent, and as the reagent temperature increases so the absorbance of the assay mixture will increase. Therefore it is important that the assay reagent is at room temperature at the beginning of the assay. However, over time dye-protein aggregates form and become sufficiently large to produce a visible precipitate that interferes with accurate spectrophotometric measurement of the dye-protein complex. This is usually not a problem within the first hour after addition of the assay reagent, but if an obvious precipitate has formed before the absorbance is measured, the assay should be repeated and the absorbance measured sooner after addition of the assay reagent. Formation of a precipitate almost immediately after addition of the assay reagent is usually the result of detergent (or other interfering substances) in the protein sample.
8. Generally, it is preferable to use a single new disposable polystyrene semi-micro cuvette that is discarded after a series of absorbance measurements. Rinse the cuvette with reagent before use, zero the spectrophotometer on the reagent blank and then do not remove the cuvette from the machine. Replace the sample in the cuvette gently using a disposable polyethylene pipet.

Table 2
Comparison of the Response of Different Proteins in the Bradford Assay

	Relative absorbance	
Protein	Assay 1	Assay 2
Myelin basic protein	139	–
Histone	130	175
Cytochrome c	128	142
Bovine serum albumin	100	100
Insulin	89	–
Transferrin	82	–
Lysozyme	73	–
α-Chymotrypsinogen	55	–
Soybean trypsin inhibitor	52	23
Ovalbumin	49	23
γ-Globulin	48	55
β-Lactoglobulin A	20	–
Trypsin	18	15
Aprotinin	13	–
Gelatin	–	5
Gramicidin S	5	–

For each protein, the response is expressed relative to that of the same concentration of bovine serum albumin. The data for Assays 1 and 2 are recalculated from ***ref. 5*** *and* ***7***, respectively.

9. The standard curve is non-linear because of problems introduced by depletion of the amount of free dye. These problems can be avoided, and the linearity of the assay improved, by plotting the ratio of absorbances at 595 and 450 nm ***(16)***. If this approach is adopted, the absolute optical density of the free dye and dye-protein complex must be determined by measuring the absorbance of the mixture at each wavelength relative to that of a cuvette containing only water (and no assay reagent). As well as improving the linearity of the calibration curve, taking the ratio of the absorbances at the two wavelengths increases the accuracy and improves the sensitivity of the assay by up to 10-fold ***(16)***.
10. For routine measurement of the protein content of many samples the microassay may be adapted for use with a microplate reader ***(7,17)***. The total volume of the modified assay is limited to 210 µL by reducing the volume of each component. Ensure effective mixing of the assay components by pipetting up to 10 µL of the protein sample into each well before adding 200 µL of the assay reagent. If a wavelength of 595 nm cannot be selected on the microplate reader, absorbance may be measured at any wavelength between 570 nm and 610 nm. However, absorbance measurements at wavelengths other than 595 nm will decrease the sensitivity of response and may increase the minimum detection limit of the protocol.

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5

Ultrafast Protein Determinations Using Microwave Enhancement

Robert E. Akins and Rocky S. Tuan

1. Introduction

In this chapter, we describe modifications of existing protein assays that take advantage of microwave irradiation to reduce assay incubation times from the standard 15–60 min down to just seconds ***(1)***. Adaptations based on two standard protein assays will be described:

1. The classic method of Lowry et al. (**ref.** *2* and *see* Chapter 2), which involves intensification of the biuret reaction through the addition of Folin-Ciocalteau phenol reagent; and
2. The recently developed method of Smith et al. ***(3)***, which involves intensification of the biuret assay through the addition of bicinchoninic acid (BCA) (*see* Chapter 3).

Performing incubations in a 2.45–GHz microwave field (i.e., in a microwave oven) for 10–20 s results in rapid, reliable, and reproducible protein determinations. We have provided here background information concerning protein assays in general and the microwave enhanced techniques in particular. The use of microwave exposure as an aid in the preparation of chemical and biological samples is well established; interested readers are directed to Kok and Boon ***(4)*** for excellent discussions concerning the application of microwave technologies in biological research.

1.1. Microwave Assay

Household microwave ovens expose materials to nonionizing electromagnetic radiation at a frequency of about 2.45 GHz (i.e., 2.45 billion oscillations/s). Such exposures have been applied to a myriad of scientific purposes including tissue fixation, histological staining, immunostaining, PCR, and many others. The

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practical and theoretical aspects of many microwave techniques are summarized by Kok and Boon ***(4),*** and the reader is directed to this reference for excellent discussions concerning general procedures and theoretical background.

Microwave ovens are conceptually simple and remarkably safe devices (*see* **Note 1**). Typically, a magnetron generator produces microwaves that are directed toward the sample chamber by a wave guide. The beam is generally homogenized by a "modestirrer," consisting of a reflecting fan with angled blades that scatter the beam as it passes. The side walls of the chamber are made of a microwave reflective material, and the microwaves are thereby contained within the defined volume of the oven (*see* **Note 2**). Specimens irradiated in a microwave oven absorb a portion of the microwave energy depending on specific interactions between the constituent molecules of the sample and the oscillating field.

As microwaves pass through specimens, the molecules in that specimen are exposed to a continuously changing electromagnetic field. This field is often represented as a sine wave with amplitude related to the intensity of the field at a particular point over time and wavelength related to the period of oscillation. Ionically polarized molecules (or dipoles) will align with the imposed electromagnetic field and will tend to rotate as the sequential peaks and troughs of the oscillating "wave" pass. Higher frequencies would, therefore, tend to cause faster molecular rotations. At a point, a given molecule will no longer be able to reorient quickly enough to align with the rapidly changing field, and it will cease spinning. There exists, then, a distinct relationship between microwave frequency and the "molecular-size" of the dipoles that it will affect. This relationship is important, and at the 2.45–GHz frequency used in conventional microwave ovens, only small molecules may be expected to rotate; specifically, water molecules rotate easily in microwave ovens but proteins do not (*see* **Note 3**).

For most microwave oven functions, a portion of the rotational energy of the water molecules in a sample dissipates as heat. Since these molecular rotations occur throughout exposed samples, microwave ovens provide extremely efficient heating, and the effects of microwaves are generally attributed to changes in local temperature. At some as yet undetermined level, however, microwave exposure causes an acceleration in the rate of reaction product formation in the protein assays discussed here. This dramatic acceleration is independent of the change in temperature, and our observations have suggested that microwave-based heating is not the principal means of reaction acceleration.

1.2. Microwave Enhanced Protein Determinations

Modifications of the procedures of Lowry (Chapter 2) and Smith (Chapter 3) to include microwave irradiation result in the generation of linear standard curves. **Figure 1** illustrates typical standard curves for examples of each assay using bovine serum albumin (BSA). Both standard curves are linear across a practical range of protein concentrations.

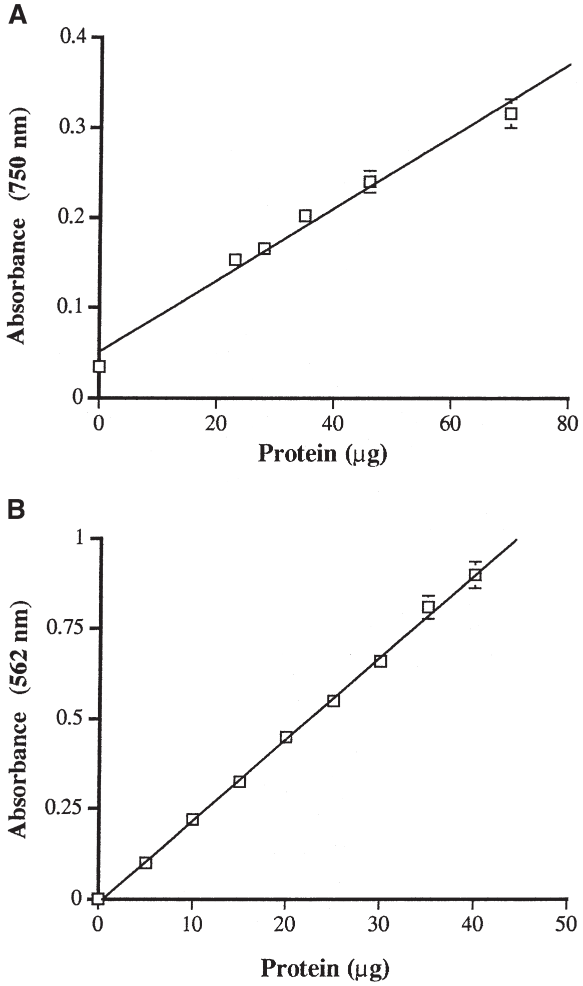

Fig. 1. Typical standard curves for microwave Lowry and BCA assays. Standard curves were generated with microwave protocols using BSA (Sigma) dissolved in water. BSA samples in 100-µL vol were prepared in triplicate for each assay and were combined with reagent as described in **Notes 1** and **2**. Tubes and a water load (total vol 100 mL, *see* **Note 5**) were placed in the center of a microwave oven. Samples for the Lowry assay were irradiated for 10 s; samples for the BCA assay were irradiated for 20 s. Results in **A** show a linear standard curve generated using a microwave Lowry assay. Results in **B** show a linear standard curve generated using a microwave BCA assay. Values presented are means ± SD.

One interesting difference between the two microwave enhanced assays concerns the relationship between irradiation time and assay sensitivity. In the *DC Protein Assay*, a modification of Lowry's *(2)* procedure supplied by Bio-Rad

Laboratories, Inc. (Hercules, CA), illustrated in **Fig. 1**, a colorimetric end point was reached after 10 s of microwave irradiation; no further color development occurred in the samples. This end point was identical to that achieved in a 15-min room temperature control assay.

The *BCA* Protein Assay*, a version of Smith's assay *(3)* supplied by Pierce Chemical Co. (Rockford, IL), afforded some flexibility in assay sensitivity because the formation of detectable reaction product was a function of the duration of microwave exposure. **Figure 2** shows the rate of reaction product formation for three different concentrations of BSA as a function of microwave exposure in a BCA assay. Absorbance values increased for each BSA concentration as a second order function of irradiation time. A linear standard curve could be generated from BSA dilutions that were irradiated for any specific time; longer irradiation times yielded more steeply sloped standard curves. In practice, then, the duration of microwave exposure can be selected to correspond to a desired sensitivity range with longer times being more suitable for lower protein concentrations. In contrast to the *DC Protein Assay*, the BCA microwave procedure described here is more sensitive than a standard, room temperature assay, and we have used it for most applications (*see* **Note 4**).

Figure 3 illustrates that the dramatic effects of microwave exposure cannot be mimicked by external heating. These results are surprising since microwave

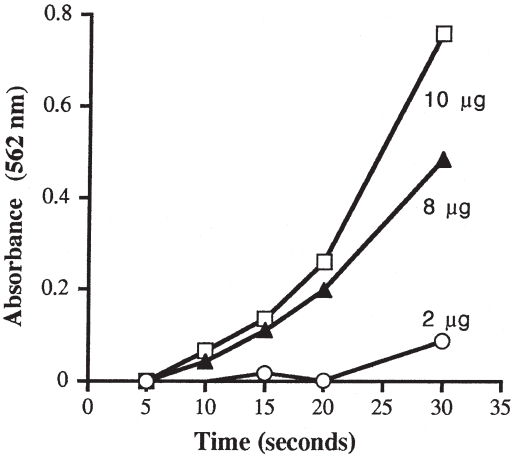

Fig. 2. Effect of Increasing microwave irradiation time on the BCA assay. Three amounts of BSA (Sigma) were prepared in water: 10 µg/tube (□), 8 µg/tube (▲), 2 µg/tube (○). Each time point was determined from triplicate samples in a single irradiation trial with the water load replaced between determinations. Each assay time resulted in the generation of a linear standard curve; the slope of each standard curve increased as a function of irradiation time. Values were normalized to the 5-s time point and presented as means ± SD.

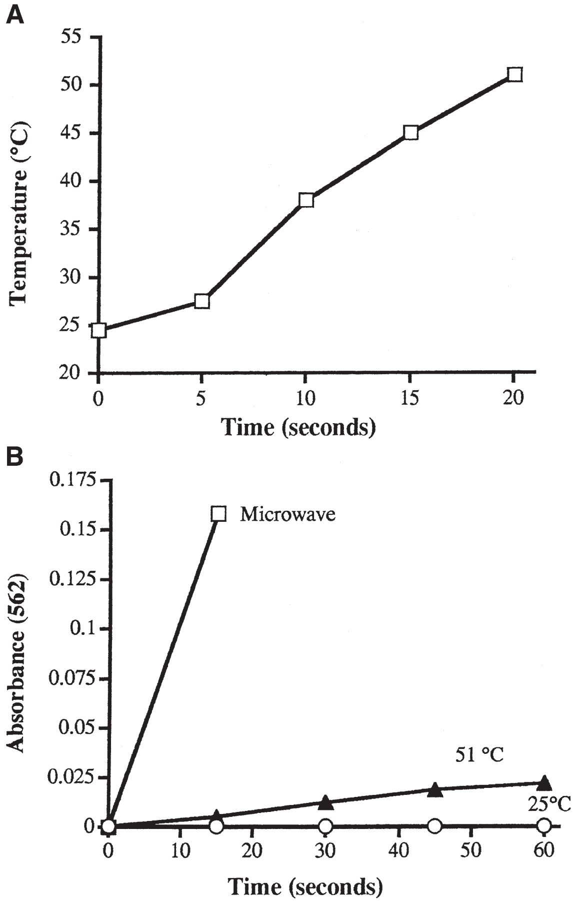

Fig. 3. Comparison of microwave irradiation with incubation at elevated temperature. (A) The change in temperature of BCA assay samples containing bovine serum albumin (BSA, Sigma). Temperatures reached 51°C during a typical 20-s irradiation. Since the assays are carried out in an open system, temperatures near 100°C would cause sample boil-over and should be avoided. (B) Development of reaction product under three different conditions: microwave irradiation (□), incubation at 51°C (▲), and incubation at 25°C (○). Incubation at 51°C, the maximal temperature reached during a 20-s irradiation, did not mirror microwave irradiation.

effects are generally attributed to increases in temperature. It is not clear at what level(s) microwaves interact with the biochemical processes involved in protein estimation; however, the acceleration is possibly related to an alteration in solvent/solute interactions. As the solvent water molecules rotate, specific structural changes may occur in the system such that interactions between solvent and solute molecules (or among the solvent molecules themselves) tend to enhance the chemical interactions between the protein and the assay components to accelerate the rate of product formation. For example, water molecules rotating in a microwave field may no longer be available to form hydrogen bonds within the solvent/solute structure. Clearly, the nature and mechanism of nonthermal microwave effects need to be studied further.

2. Materials

2.1. Lowry Assay (see Chapter 2)

Lowry reagents are available from commercial sources. Assay reagents were routinely purchased from Bio-Rad Laboratories in the form of a detergent-compatible Lowry kit (*DC Protein Assay*). Assay reactions are typically carried out in polystyrene Rohren tubes (Sarstedt, Inc., Newtown, NC). Tubes were placed in a plastic test tube rack at the center of a suitable microwave oven (*see the following*) along with a beaker containing approx 100 mL of H_2O (*see* **Note 5**).

2.2. BCA Assay (see Chapter 3)

BCA protein reagent is available from commercial sources. Assay reagents are routinely purchased from Pierce Chemical Co. in the form of a *BCA* Protein Assay* kit. As with the Lowry assay, reactions were typically carried out in polystyrene Rohren tubes (Sarstedt). Tubes were placed in a plastic test tube rack at the center of a suitable microwave oven (*see* **Subheading 3.2**) along with a beaker containing approx 100 mL of H_2O (*see* **Note 5**).

3. Methods

3.1. Sample Preparation

Sample preparation should be carried out as specified by the manufacturers. Generally, samples are solubilized in a noninterfering buffer (*see* **Note 3**) so that the final protein concentration falls within the desired range (*see* **Note 4**). Samples should be either filtered or centrifuged to remove any debris prior to protein determination.

3.2. Selection of Microwave Oven

Microwave ovens differ substantially in their suitability for these assays. Desirable attributes include fine control of irradiation time, a chamber size large enough to easily accommodate the desired number of samples, and a configuration that results in a homogenous field of irradiation so that all samples within the central volume of the oven receive a uniform microwave dose (*see* **Note 4**). Samples should be placed in a nonmetallic test tube rack in the center of the oven. A volume of room temperature water is included in the oven chamber as well so that the total amount of fluid (samples + additional water) is constant from one assay to another (*see* **Note 5**).

3.3. Sample Irradiation

Once the samples are placed into the center of the microwave chamber, close the door and irradiate the samples. Using the *DC Protein Assay*, a 10-s irradiation was optimal as a replacement for the standard 15–min incubation. Using the *BCA* Protein Assay*, a 20-s irradiation has proved adequate in most situations. We have found it most convenient to use the highest setting on the microwave oven and to control exposure using an accurate timer.

3.4. Reading and Interpreting Assay Results

After irradiation, the absorbance of each sample and standard should be determined spectrophotometrically. Samples from Lowry assays should be read at 750 nm and samples from BCA assays should be read at 562 nm. A standard curve of absorbance values as a function of standard protein concentration can be generated easily and used to determine the protein levels in the unknown samples. It is recommended that standard curves be generated along with each assay to avoid any difficulties that may arise from differences in reagents or alterations in total microwave exposure.

3.5. Summary

In summary, the microwave BCA protein assay protocol is as follows:

1. Combine samples and BSA standards with BCA assay reagent in polystyrene tubes;
2. Place samples into an all plastic test tube rack in the center of a microwave oven along with a beaker containing a volume of room temperature water sufficient to make the total volume of liquid in the chamber 100 mL;
3. Irradiate samples for 20 s on the highest microwave setting; and
4. Measure A_{562} for each sample and determine protein concentrations based on a BSA calibration curve.

The microwave Lowry assay protocol is virtually identical:

1. Combine samples and Lowry assay reagents in polystyrene tubes;
2. Place samples into an all plastic test tube rack in the center of a microwave oven along with a beaker containing a volume of room temperature water sufficient to make the total volume of liquid in the chamber 100 mL;
3. Irradiate samples for 10 s on the highest microwave setting; and
4. Measure A_{750} for each sample and determine protein concentrations based on a BSA calibration curve.

Microwave protein assays are suitable for all situations where standard assays are presently used. The ability to determine accurate protein concentrations in so little time should greatly facilitate routine assays and improve efficiency when protocols require protein determination at multiple intermediate steps. Similar microwave techniques have also been applied as time-saving and efficiency-enhancing procedures by several authors (*see* **ref.** *4*). We have used microwave assays to generate chromatograms during protein purification and for general protein determinations (e.g., before electrophoretic analysis). The assays consistently yield reliable results that are comparable to those obtained by standard protocols. The modifications we present here are very easily adapted to most commercially available microwave ovens and, in the case of the BCA assay, can be adjusted to cover a wide range of protein concentrations. Since the duration of the assays is so short, it is possible to try several irradiation times and water loads to select the specific conditions required by the particular microwave and samples to be used. Microwave enhanced protein estimations should prove to be extremely useful in laboratories currently doing standard Smith ***(3)*** or Lowry ***(2)*** based protein determinations.

4. Notes

1. Although microwave radiation is nonionizing, precautions should be taken to avoid direct irradiation of parts of the body. Microwaves can penetrate the skin and cause significant tissue damage in relatively short periods of time. Most contemporary microwave ovens are remarkably safe and leakage is unlikely; however, periodic assessment of microwave containment within the microwave chamber should be carried out. Perhaps more dangerous than radiation effects are potential problems caused by the rapid heating of irradiated samples. Care should be taken when removing samples from the microwave chamber to avoid getting burned, sealed containers should not be irradiated as they may explode, and metal objects should be excluded from the chamber to avoid sparking. Users should consult their equipment manuals and institute safety offices prior to using microwave ovens. A detailed discussion of microwave hazards is included in **ref.** *4*.
2. Microwave ovens use a nominal frequency of 2.45 GHz. The energy put into the microwave chamber is actually a range of frequencies around 2.45 GHz. As the waves in the oven reflect off of the metal chamber walls, “hot” and “cold” spots may be set up by the constructive and destructive interference of the waves. The positions of these “hot” and “cold” spots is a function of the physical design of the oven chamber and the electrical properties of the materials contained in the oven. The unevenness of microwave fields can be minimized by mode stirring *(see* the preceeding*),* appropriate oven configuration, or by rotating the specimen in the chamber during irradiation. If the design of a particular microwave oven does not provide a relatively uniform irradiation volume, it may not be useful for the assays outlined here.

The suitability of a particular microwave oven may be tested easily using a number of tubes with known concentrations of protein. Uneven irradiation patterns will be detected by significant differences in the color development for a given protein concentration as a function of position within the oven chamber. We have had success with several microwave ovens including a 0.8 cu ft, 600 W, General Electric oven, model JEM18F001 and a 1.3 cu ft, 650 W, Whirlpool oven, model RJM7450.

3. The frequency used in household microwave ovens is actually below the resonance frequency for water. Above the 2.45 GHz used in household microwave ovens, water molecules are capable of rotating faster and of absorbing substantially more microwave energy. Too much absorption, however, is undesirable. It is possible that the outer layers of a sample may absorb energy so efficiently that the interior portions receive substantially less energy. The resulting uneven exposure may have adverse effects.

   It is important to note that molecules other than water may absorb microwave energy. If a compound added to a microwave enhanced assay absorbs strongly near 2.45 GHz, uneven sample exposures may result because of the presence of the compound. In addition, compounds that are degraded or converted during a microwave procedure, or compounds that have altered interactions with other assay components during irradiation, may substantially affect assay results. Although it may be possible to predict which materials would interfere with a given assay by considering the relevant chemical and electrical characteristics of the constituent compounds, potential interference is most easily assessed empirically by directly determining the effects of a given additive on the accuracy and sensitivity of the standard microwave assay.
4. Assay sensitivity may be improved by increasing irradiation time. The time required may be determined quickly by using BSA test solutions in the range of protein concentrations expected until desirable A_{562} values were obtained. By increasing microwave exposure time, it is possible to substantially increase assay sensitivity while keeping irradiation times below 60 s. The ease with which sensitivity may be adjusted within extremely short time frames places the microwave BCA assay among the quickest, most flexible assays available for protein determinations.
5. The addition of a volume of water to the microwave chamber, such that the total volume contained in the microwave chamber is constant from one assay to the next, allows irradiation conditions to be controlled easily from assay to assay. The additional water acts as a load on the oven and absorbs some of the microwave energy. Since the total amount of water remains constant from one assay to the next, the amount of

energy absorbed also remains constant. The time of irradiation, therefore, becomes independent of the number of samples included in the assay.

Acknowledgments

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6

The Nitric Acid Method for Protein Estimation in Biological Samples

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1. Introduction

1.1. Background

The quantitation of protein in biological samples is of great importance and utility in many research laboratories. Protein measurements are widely utilized to ensure equal loading of samples on sodium dodecyl sulfide (SDS)-polyacrylamide gels and to provide a basis for comparison of enzyme activities and other analytes.

Several methods are commonly employed for the determination of protein content in biological samples, including the measurement of absorbance at 260 ***(1)*** or 205 nm (***2***, and Chapter 1); the method of Lowry et al. (***3***, and Chapter 2), which relies on the generation of a new chromophore on reaction of an alkaline protein hydrolysate with phosphotungstic-phosphomolybdic acid in the presence of Cu^{2+} ***(4)***; the method of Smith et al. (***5***, and Chapter 3), which relies upon the reduction of Cu^{2+} to Cu^{+}, which then forms a colored complex with bicinchoninic acid (BCA); the method of Bradford (***6***, and Chapter 4), which relies on a change in absorbance on binding of Coomassie Blue to basic and aromatic amino acids under acidic conditions ***(7)***; and the method of Böhlen et al. ***(8)***, which relies on the reaction of fluorescamine with primary amines to generate a fluorescent product. Unfortunately, compounds that are constituents of biological buffers sometimes interfere with these methods, limiting their application.

Nucleic acids, neutral detergents of the polyoxyethylenephenol class, SDS, and urea interfere with spectrophotometric determinations at 260 and 205 nm ***(9)***. Several components commonly used in biological buffers, including

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tris-hydroxymethyl-amino methane (Tris), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic [acid] (HEPES), ethylenediaminetetraacetic acid (EDTA), neutral detergents, and reducing agents, interfere with the method of Lowry et al. ***(10)***. In addition, Tris, ammonium sulfate, EDTA, and reducing agents interfere with the BCA method, although neutral detergents and SDS do not ***(5)***. Finally, many commonly used reagents, including neutral detergents and SDS, interfere with the method of Bradford ***(6)***. Details of compounds that interfere with these assays can be found in **Table 1**, Chapter 3.

Several approaches have been proposed to circumvent the aforementioned difficulties. Protein precipitation with deoxycholate and trichloroacetic acid will eliminate many interfering substances ***(10)***, but the inclusion of this step is laborious when multiple samples are being analyzed simultaneously. Alternatively, samples can be lysed in a buffer that is compatible with a particular assay, then diluted into a second buffer after analysis. For example, cells lysed in Triton X-100 can be assayed for protein and then diluted into SDS sample buffer in preparation for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This approach, however, can lead to false results, as illustrated by the demonstration that caspase-3 activation in lysates of mitogen-stimulated lymphocytes reflects proteolytic cleavage occurring in the cell lysate rather than in the intact cells prior to lysis ***(11, 12)***. Finally, protein can be estimated by solubilizing samples directly in SDS sample buffer, spotting them onto a solid support (e.g., glass or nitrocellulose), washing away interfering substances, reacting the immobilized polypeptides with Coomassie Blue, and eluting the Coomassie Blue to estimate the protein content ***(13, 14)***, but this process is time and labor intensive.

As a result of these shortcomings, there is still a need for alternative methods of protein determination in biological samples. The nitric acid method detailed in this chapter represents one such method ***(15)***. This method is based upon studies dating to the 1800s demonstrating that aromatic molecules undergo nitration when treated with nitric acid. In particular, treatment of tyrosine with nitric acid produces 3-nitrotyrosine ***(16)***, which is distinguished from the parent compound by the appearance of a new absorbance peak at 358 nm. The method detailed in this chapter utilizes this chemistry to create a one-step method for protein determination in biological samples.

1.2. Nitric Acid Method for Protein Determination

1.2.1. Technique Derivation

Our laboratory has frequently examined the cellular accumulation of cytotoxic therapeutic drugs ***(17, 18)***. Because of concern about drug efflux from cells during the course of manipulation (e.g., trypsinization and centrifugation), we sought a method for the assessment of protein content after cells were washed *in situ* and then solubilized directly in nitric acid, a matrix used for platinum-DNA adduct determination.

During the course of solubilizing cells in nitric acid, we noted a brownish color change that varied in intensity with the quantity of cells. Examination of the absorbance spectrum of these nitric acid lysates demonstrated a peak at 358 nm (**Fig. 1A**, inset) that was not present in cells solubilized in SDS sample buffer. Comparison of two human leukemia cell lines, HL-60 (diameter 11 μm) and K562 (diameter 15 μm), revealed that the absorbance at 358 nm increased linearly with cell number and that the slope was two-fold higher in the larger cell line (**Fig. 1A**). We hypothesized that the new absorbance peak at 358 nm reflected a reaction of nitric acid with one or more amino acids. While nitric acid solubilization of L-tyrosine produced a sharp peak at 358 nm and similar treatment of L-tryptophan produced a shoulder at the same wavelength, other amino acids, including L-serine, L-arginine, L-phenylalanine, L-threonine, L-alanine, L-proline, L-valine, L-histidine, L-methionine, L-isoleucine, L-glutamic acid, L-aspartic acid and L-asparagine failed to yield products that absorbed at 358 nm (**Fig. 1B** and data not shown). Nucleotides (ATP, CTP, GTP, TTP, UTP), RNA, and DNA also failed to yield products that absorbed at 358 nm (data not shown). These investigations led us to propose that the 358 nm absorbance peak observed after nitric acid solubilization of cells primarily reflects the tyrosine content of cellular proteins.

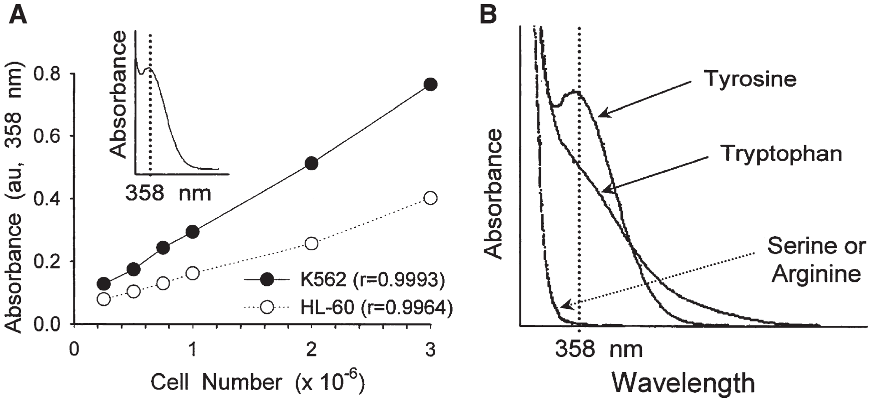

Fig. 1. Treatment of cells or L-tyrosine with nitric acid results in an increase in absorbance at 358 nm. **(A)** Relationship between cell number and absorbance at 358 nm in HL-60 and K562 cells solubilized in nitric acid for 24 h. *Inset*, absorbance spectrum resulting from treatment of OV202 cells with nitric acid. **(B)** Absorbance spectra resulting from treatment of L-tyrosine, L-tryptophan, L-serine, or L-arginine with nitric acid for 1 h. Amino acid concentrations in nitric acid were all 0.1 mg/mL.

To better understand the chemistry responsible for these observations, we solubilized L-tyrosine in nitric acid for 1 h. A single reaction product was formed that co-migrated with authentic 3-nitrotyrosine by high performance liquid chromatography (HPLC) and had an absorbance maximum at 358 nm (**Fig. 2**). Incubation for prolonged periods of time resulted in disappearance of

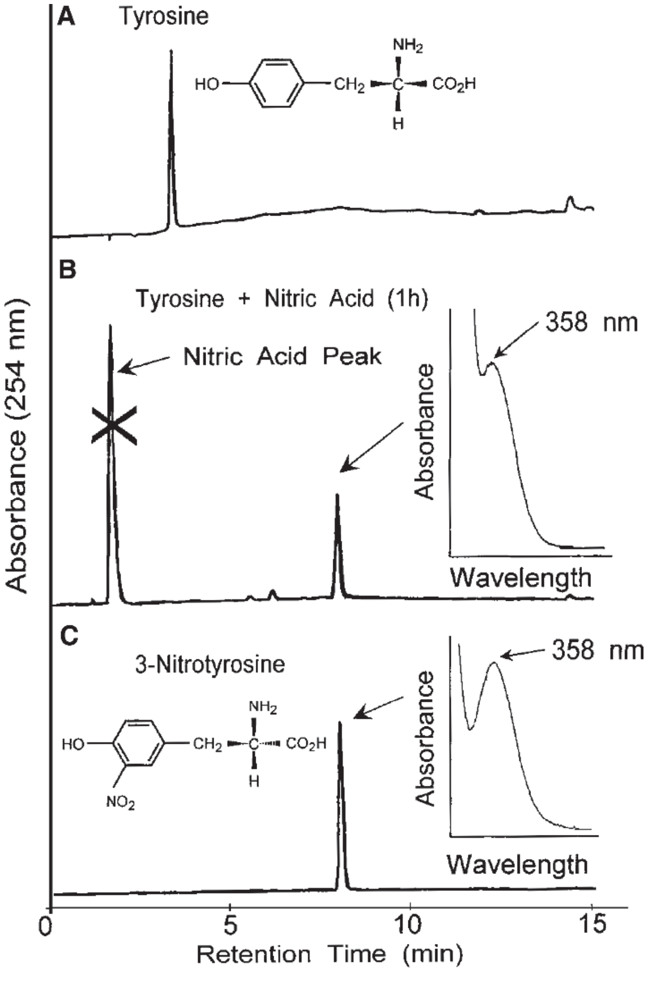

Fig. 2. Nitrotyrosine results from the treatment of L-tyrosine with nitric acid. **(A)** HPLC chromatogram of L-tyrosine. **(B)** HPLC chromatogram of L-tyrosine reacted with nitric acid for 1 h. *Inset*, absorbance spectrum of peak eluting at ~ 8 min. The prominent peak eluting at 2 min corresponds to a contaminant in the nitric acid. **(C)** HPLC chromatogram of 3-nitrotyrosine. *Inset*, absorbance spectrum of peak eluting at ~8 min. HPLC was accomplished using a Beckman Ultrasphere obs column (4.6 × 250 mm, 5 µm) as previously described *(15)*.

the 3-nitrotyrosine peak and appearance of additional peaks that are thought to reflect nitration at additional sites as well as oxidation (data not shown). As a consequence, the absorbance at 358 nm reached a peak between 1 and 6 h, and then subsequently declined.

To determine whether these observations could be utilized to devise a method for quantitating protein, bovine serum albumin (BSA) was solubilized in nitric acid and incubated for varying lengths of time. As indicated in **Fig. 3A**, absorbance at 358 nm measured after incubation for 0.5–70 h was a linear function of protein content. Examination of these data revealed that production of the chromophore in response to nitric acid treatment at room temperature was approximately 88% complete within 30 min, reached a maximum at approximately 24 h, and remained relatively stable thereafter (**Fig. 3A**, inset). We hypothesize that the relative stability of the 358 nm peak in proteins treated for prolonged periods with nitric acid (in contrast to the disappearance of the same peak when L-tyrosine is subjected to prolonged nitric acid treatment) results from diminished susceptibility of tyrosyl residues in protein to react beyond 3-nitration.

To determine whether this approach could be utilized to quantitate protein under conditions compatible with SDS-PAGE, aliquots of BSA in SDS sample buffer containing Tris, SDS, urea, and β-mercaptoethanol were treated with nitric acid. As indicated in **Fig. 3B**, the presence of increasing amounts of protein resulted in increasing absorbance at 358 nm, which was again a linear function of protein content. To confirm that the present assay was useful for measuring polypeptides other than BSA, we assessed absorbance after reaction of five other polypeptides with nitric acid including bovine insulin, human fibrinogen, human fibronectin, chicken ovalbumin, and rat albumin. Each of these polypeptides reacted with nitric acid to produce species with absorbance maxima at 358 nm. In each case, there was a strong correlation ($r > 0.99$) between protein content and absorbance (data not shown).

1.2.2. Interfering Substances

Urea, SDS, β-mercaptoethanol, 20% glycerol, and 50% saturated ammonium sulfate did not interfere with protein determination by the nitric acid method. In addition, the neutral detergents Tween 20 (1%), Brij 97 (1%), *n*-octyl α-D-glucopyranoside, and digitonin (10 μ*M*) did not interfere with this method. In contrast, the presence of 1% Triton X-100 (a phenol derivative) or trace amounts of phenol produced strong absorbance at 358 nm that interfered with the assay. Although 1% 3-([3-cholamidopro-pyl]dimethylammonio)-1-propanesulfonate (CHAPS) increased absorbance at 358 nm, it did not interfere with the assay - as long as the protein samples used to generate standard curves were also solubilized in CHAPS.

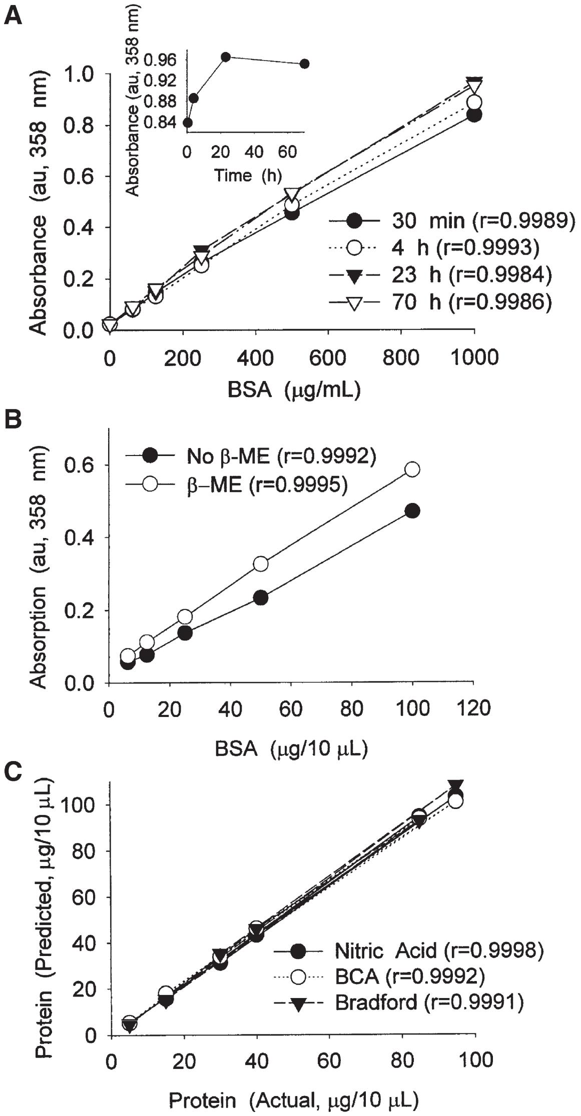

Fig. 3. Measurement of absorbance at 358 nm can be used to quantitate protein levels in samples treated with nitric acid. **(A)** Absorbance (at 358 nm) of varying concentrations of BSA incubated in nitric acid for 30 min, 4 h, 23 h, and 70 h. Inset, time-dependent increase of absorbance at 358 nm for a 1000 µg/mL solution of BSA dissolved in nitric acid. **(B)** Relationship between BSA concentration and absorbance at 358 nm for samples dissolved in SDS-PAGE sample buffer with or without 5% (v/v) β-mercaptoethanol. **(C)** Relationship between actual and measured BSA concentration in six blindly submitted BSA samples assessed by the nitric acid, BCA, and Bradford protein assays. Results in **B** and **C** represent the means of results from the assay of triplicate samples. Error bars (hidden by data points) represent ± 1 sample standard deviation.

1.2.3. Comparison of the Nitric Acid Method to Bradford and BCA Protein Assays

To assess the reliability of this technique, protein content was blindly measured in six unknown samples using the Bradford assay (Coomassie Blue binding), BCA assay, and the nitric acid method. Results of these experiments indicated that the three methods produce comparable results in assessing protein content in unknown samples (**Fig. 3C**). The correlation between actual and predicted protein content was slightly higher for the nitric acid method ($r = 0.9998$) than for the BCA assay ($r = 0.9992$) or Bradford assay ($r = 0.9991$). We also examined whether the nitric acid method is suited to comparing protein levels between different cell lines. To assess this possibility, we ran SDS-PAGE gels with extracts from five different human cancer cell lines loaded in accord with the amounts of protein in the extracts indicated by either the BCA or the nitric acid method. When we compared Western blots for a number of different proteins, we found that both protein assays facilitated similar gel loading (**Fig. 4**). It should be noted, however, that the nitric acid method uniformly resulted in proportionately higher protein values (and hence proportionately less cell extract loaded per gel lane) in comparison to the BCA method.

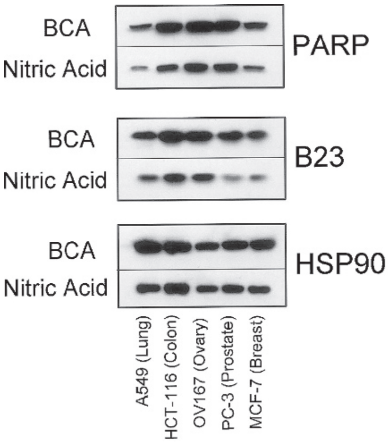

Fig. 4. BCA and nitric acid protein assays produce equivalent results when used as the basis for loading SDS-PAGE gels with extracts from different cell lines. Extracts from five human cancer cell lines (each containing 50 µg of total cellular proteins, as assessed by the BCA or the nitric acid method) were subjected to SDS-PAGE and probed for PARP, B23, and HSP90 via Western blotting. All shown pairs of BCA/nitric acid immunoblots were derived from the same gel and have been aligned to facilitate comparison.

2. Materials

1. 70% Nitric acid, store in acid cabinet at room temperature; CAUTION: toxic, use in fume hood and avoid inhalation of fumes and skin contact.
2. Bovine serum albumin (BSA), storage at 4°C.
3. UV-vis spectrophotometer.
4. (Optional) SDS sample buffer for cell lysis: 2% (w/v) SDS, 4 M urea (deionized with Bio-Rad AG501-X8 mixed bead resin prior to use), 62.5 mM Tris-HCl, pH6.8, 1mM EDTA, and 5% (v/v) β-mercaptoethanol.

3. Methods

3.1. Nitric Acid Protein Determination Method: Cell Lysates

The following is the recommended procedure for determining protein content in samples solubilized in SDS sample buffer or other detergent-based cell lysis buffers without bromophenol blue added ***(21–22)***.

1. Prior to assay for protein, cells must be washed in serum-free medium or 1X PBS to remove exogenous protein and cross-reacting substances contained in culture media.
2. Lyse cells directly in SDS sample buffer or desired detergent-based cell lysis buffer.
3. Remove an aliquot of cell lysate expected to contain 5–100 μg of protein. Bring sample volume to 10 μL using same buffer samples were lysed in (*see* **Note 1**).
4. In a fume hood, add 140 μL 70% nitric acid to bring total volume to 150 μL (*see* **Note 2**).
5. Incubate at room temperature for 2 h (*see* **Note 3**).
6. Utilizing glass or quartz microcuvettes, or a 96-well plate (*see* **Note 2)**, measure absorbance at 358 nm with H_2O as a blank (*see* **Note 4**).
7. Compare results to a standard curve constructed in parallel using varying amounts of BSA in 10 μL SDS sample buffer subjected to the same conditions (*see* **Notes 6** and **7**).

3.2. Nitric Acid Protein Determination Method: Whole Cells

Simple modifications allow this procedure to be applied to other settings. This variation is especially helpful when protein content in whole cells is to be determined in conjunction with assessment of cellular drug accumulation ***(17–20)***.

1. Wash cells thoroughly with serum-free buffer to remove exogenous proteins contained in culture media.
2. Solubilize cells directly in 70% nitric acid.
3. Incubate at room temperature for 24 h (*see* **Note 5**).
4. Remove an aliquot for determination of absorbance at 358 nm (dilution in 70% nitric acid may be necessary to assure that all results fall within the linear range of assay, *see* **Note 2**) with H_2O as a blank (*see* **Note 4**).

5. Compare results to a standard curve constructed in parallel using varying amounts of BSA subjected to the same assay conditions (*see* **Notes 6** and **7**).

3.3. Nitric Acid Protein Determination Method: Perchloric or Trichloroacetic Acid Precipitates

When perchloric acid or trichloroacetic acid precipitates are assayed for protein content:

1. Solubilize precipitates directly in nitric acid.
2. Incubate for 2 h at room temperature (*see* **Note 3**).
3. Determine absorbance at 358 nm (dilution in 70% nitric acid may be necessary, *see* **Note 2**) with H_2O as a blank (*see* **Note 4**).
4. Compare results to a standard curve constructed in parallel using varying amounts of BSA subjected to the same assay conditions (*see* **Notes 6** and **7**).

4. Notes

1. One advantage of the nitric acid method is that protein content can be assessed using samples solubilized in SDS sample buffer immediately prior to loading samples for electrophoresis. It is possible to solubilize samples directly in SDS sample buffer, remove an aliquot for protein determination, and then adjust the sample volume based on the result of the assay. Bromophenol blue for gel visualization should only be added to the samples after removing an aliquot to determine protein concentration, as it is not compatible with the assay.
2. 150 µL volumes are suggested for measuring in a microcuvette. If desired, volumes of sample and nitric acid may be adjusted up proportionately to be read in a larger cuvette. Also, the assay may be adapted to a microplate format using a quartz or glass 96-well plate using a UV-visible microplate reader at 358 nm.
3. Incubations are routinely performed between 20–22°C. Incubation at 37°C revealed that the reaction was not appreciably altered (data not shown) and is therefore relatively temperature insensitive.
4. Our experience indicates that H_2O, rather than 70% nitric acid, should be used for a blank for all spectrophotometric determinations.
5. The assay of protein content in detergent-solubilized cells by the nitric acid method requires an incubation period of only 2 h at 22°C. In contrast, similar determinations on nitric acid-solubilized cells require incubation periods of at least 24 h for optimal detection of differences between different cell lines. Longer incubations may be required for nitric acid-solubilized cells because longer times may be required to complete protein nitration under these conditions.

6. Proteins with differing tyrosine/amino acid content would be expected to yield different slopes when utilized as standards for this assay. Although this can present a problem in experiments that require the precise quantitation of a single polypeptide species, it does not appear to be a limitation when this method is used to compare relative protein content in complex biological mixtures. Moreover, when the nitric acid assay was applied to a variety of polypeptides (including BSA, rat albumin, chicken ovalbumin, bovine insulin, human fibrinogen, and human fibronectin), the resulting slopes did not correlate with either tyrosine or tryptophan content of the polypeptides (data not shown), suggesting that tertiary structure or other factors might also influence the extent of nitration during nitric acid treatment. Similar differences in reactivity of various purified polypeptides have been previously observed with other protein estimation methods as well ***(5, 6)***.
7. The nitric acid method has a sensitivity limit of approximately 5 μg when performed using 100 μL cuvettes. This sensitivity is slightly lower than the methods of Lowry et al. ***(3)***, Smith et al. ***(5)*** and Bradford ***(6)*** performed under similar semimicro conditions.

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7

Quantitation of Tryptophan in Proteins

Alastair Aitken and Michèle P. Learmonth

1. Introduction

1.1 Hydrolysis Followed by Amino Acid Analysis

Accurate measurement of the amount of tryptophan in a sample is problematic, as it is completely destroyed under normal conditions employed for the complete hydrolysis of proteins. Strong acid is ordinarily the method of choice, and constant boiling 6 *M* hydrochloric acid is most frequently used. The reaction is usually carried out in evacuated sealed tubes or under nitrogen at 110°C for 18–96 h. Under these conditions, peptide bonds are quantitatively hydrolyzed (although relatively long periods are required for the complete hydrolysis of valine, leucine, and isoleucine). As well as complete destruction of tryptophan, small losses of serine and threonine occur, for which corrections are made. The advantages of amino acid analysis include the measurement of absolute amounts of protein, provided that the sample is not contaminated by other proteins. However, it may be a disadvantage if an automated amino acid analyzer is not readily available.

Acid hydrolysis in the presence of 6 *N* HCl, containing 0.5–6% (v/v) thioglycolic acid at 110°C for 24–72 h *in vacuo* will result in greatly improved tryptophan yields ***(1)***, although most commonly, hydrolysis in the presence of the acids described in **Subheading 3.1** may result in almost quantitative recovery of tryptophan.

Alkaline hydrolysis followed by amino acid analysis is also used for the estimation of tryptophan. The complete hydrolysis of proteins is achieved with 2–4 *M* sodium hydroxide at 100°C for 4–8 h. This is of limited application for routine analysis, because cysteine, serine, threonine, and arginine are destroyed in

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the process and partial destruction by deamination of asparagine and glutamine to aspartic and glutamic acids occurs.

The complete enzymatic hydrolysis of proteins (where tryptophan would be quantitatively recovered) is difficult, because most enzymes attack only specific peptide bonds rapidly. Often a combination of enzymes is employed (such as Pronase), and extended time periods are required (*see* Chapter 106). A further complication of this method is possible contamination resulting from autodigestion of the enzymes. Kuyama and coworkers ***(2)*** have described a method for quantification and sequence identification of tryptophan in individual proteins in complex mixtures. By labeling tryptophan residues with 2-nitrobenzenesulfenyl chloride (NBSCl) in conjunction with tandem mass spectrometry a 6Da mass differential is generated using $^{13}C_6$-labeled NBSCl and $^{12}C_6$-labeled NBSCl in two separate samples. This 6 Da mass difference between the $^{13}C_6$-labeled and the $^{12}C_6$-labeled peptides provides a mass signature to all tryptophan-containing peptides in proteolytic digests for proteomic identification.

1.2. Measurement of Tryptophan Content by UV

The method of Goodwin and Morton ***(3)*** is described in **Subheading 3.3**.

The absorption of protein solutions in the UV is due to tryptophan and tyrosine (and to a very minor, and negligible, extent phenylalanine and cysteine). The absorption maximum will depend on the pH of the solution, and spectrophotometric measurements are usually made in alkaline solutions. Absorption curves for tryptophan and tyrosine show that at the points of intersection, 257 and 294 nm, the extinction values are proportional to the total tryptophan + tyrosine content. Measurements are normally made at 294.4 nm, as this is close to the maximum in the tyrosine curve (where $\Delta\varepsilon/\Delta\varepsilon$, the change in extinction with wavelength, is minimal), and in conjunction with the extinction at 280 nm (where $\Delta\varepsilon/\Delta\lambda$ is minimal for tryptophan) the concentrations of each of the two amino acids may be calculated (*see* **Notes 1** and **2**).

2. Materials

1. 3 *M p*-Toluenesulfonic acid.
2. 0.2% 3-(2-Aminoethyl) indole.
3. 3 *M* Mercaptoethanesulfonic acid (Pierce-Warriner).
4. 1 *M* NaOH.

3. Methods

3.1. Quantitation of Tryptophan by Acid Hydrolysis

1. To the protein dried in a Pyrex glass tube (1.2 × 6 cm or similar, in which a constriction has been made by heating in an oxygen/gas flame) is added 1 mL of 3 *M p*-toluenesulfonic acid, containing 0.2% tryptamine [0.2% 3-(2-aminoethyl) indole] ***(4)***.

2. The solution is sealed under vacuum and heated in an oven for 24–72h at 110°C, *in vacuo*.
3. Alternatively, the acid used may be 3 *M* mercaptoethanesulfonic acid. The sample is hydrolyzed for a similar time and temperature ***(5)***.
4. The tube is allowed to cool, and cracked open with a heated glass rod held against a horizontal scratch made in the side of the tube.
5. The acid is taken to near neutrality by carefully adding 2 mL of 1 *M* NaOH. An aliquot of the solution (which is still acid) is mixed with the amino acid analyzer loading buffer.
6. Following this hydrolysis, quantitative analysis is carried out for each of the amino acids on a suitable automated instrument.

3.2. Alkaline Hydrolysis

1. To the protein dried in a Pyrex glass tube (as described in **Subheading 3.1., step 1**), 0.5mL of 3 *M* sodium hydroxide is added (*See* **Note 3**).
2. The solution is sealed under vacuum and heated in an oven for 4–8 h at 100°C, *in vacuo*.
3. After cooling and cracking open, *while one is wearing safety goggles*, the alkali is neutralized carefully with an equivalent amount of 1 *M* HCl. An aliquot of the solution is mixed with the amino acid analyzer loading buffer and analyzed (as described in **Subheading 3.1., step 6**).

3.3. Measurement of Tryptophan Content by UV

1. The protein is made 0.1 *M* in NaOH.
2. Measure the absorbance at 294.4 nm and 280 nm in cuvettes transparent to this wavelength (i.e. quartz) in a spectrometer (*see* **Note 4**).
3. The amount of tryptophan (w) is estimated from the relative absorbances at these wavelengths by the method of Goodwin and Morton ***(3)*** shown in **Eq. 1**., where

x = total mole/L; w = tryptophan mole/L; $(x - w)$ = tyrosine mole/L.

ε_y = Molar extinction of tyrosine in 0.1 M alkali at 280 nm = 1576.

ε_w = Molar extinction of tryptophan in 0.1 M alkali at 280 nm = 5225.

Also, x is measured from $E_{294.4}$ (the molar extinction at this wavelength). This is 2375 for both Tyr and Trp (since their absorption curves intersect at this wavelength). An accurate reading of absorbance at one other wavelength is then sufficient to determine the relative amounts of these amino acids.

$$E_{280} = w\varepsilon_w + (x - \mathrm{w})\varepsilon_y \qquad (1)$$

$$\text{Therefore}, w = (E_{280} - x\varepsilon_y) / (\varepsilon_w - \varepsilon_y)$$

4. An alternative method of obtaining the ratios of Tyr and Trp is to use the formulae (**Eq. 2**) derived by Beaven and Holiday ***(6)***.

$$M_{Tyr} = (0.592\,K_{294} - 0.263\,K_{280}) \cdot 10^{-3} = (0.263\,K_{280} - 0.170\,K_{294}) \cdot 10^{-3}$$

where M_{Tyr} and M_{Trp} are the moles of tyrosine and tryptophan in 1 g of protein, and K_{294} and K_{280} are the extinction coefficients of the protein in 0.1 *N* alkali at 294 and 280 nm. Extinction values can be substituted for the *K* values to give the molar ratio of tyrosine to tryptophan according to the formula:

$$M_{Tyr} / M_{Trp} = (0.592\ E_{294} - 0.263\ E_{280}) / (0.263\ E_{280} - 0.170\ E_{294}) \qquad (2)$$

4. Notes

1. The extinction of nucleic acid in the 280 nm region may be as much as 10 times that of protein at the same wavelength and hence a few percent of nucleic acid can greatly influence the absorption.
2. In this analysis, the tyrosine estimate may be high and that of tryptophan low. If amino acid analysis indicates absence of tyrosine, tryptophan is more accurately determined at its maximum, 280.5 nm.
3. Absorption by most proteins in 0.1 *M* NaOH solution decreases at longer wavelengths into the region 330–450 nm where tyrosine and tryptophan do not absorb. Suitable blanks for 294 and 280 nm are therefore obtained by measuring extinctions at 320 and 360 nm and extrapolating back to 294 and 280 nm.
4. In proteins, in a peptide bond, the maximum of the free amino acids is shifted by 1–3 nm to a longer wavelength and pure peptides containing tyrosine and tryptophan residues are better standards than the free amino acids. A source of error may be due to turbidity in the solution and if a protein shows a tendency to denature, it is advisable to treat with a low amount of proteolytic enzyme to obtain a clear solution.

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8

Kinetic Silver Staining of Proteins

Douglas D. Root and Kuan Wang

1. Introduction

Silver staining methods have long been know to provide highly sensitive detection of proteins and nucleic acids following electrophoresis in agarose and polyacrylamide gels ***(1,2)***. Silver staining technologies can be extended to other media such as blots, thin-layer chromatography (TLC), and microtiter plates. The quantification of proteins adsorbed to microtiter plate wells provides quantitative information for enzyme-linked immunosorbent assay (ELISA) and protein interaction assays. One nonradioactive procedure, copper iodide staining, is described in Chapter 72 ***(3)***. The kinetic silver staining method for measuring the amount of adsorbed protein in a microtiter plate has been developed recently. The microtiter plate assay has a sensitivity similar to copper iodide staining (5–150 ng/well) but higher precision (<5%; ***4***). When quantification is based on the time required for staining to reach a fixed optical density, very little protein-to-protein variation is observed, so a standard protein for calibration can be selected (such as bovine albumin) that is free of interfering substances to which the assay is sensitive ***(4)***. Furthermore, this kinetic silver staining assay is found to be most sensitive for the detection of proteins on cellulose such as is commonly used for TLC (*see* Chapter 72 for a comparison with other solid-phase stains).

2. Materials

1. Polystyrene 96-well microtiter plates (e.g., Titertek 76-381-04, McLean, VA).
2. The kinetic silver staining reagent consists of

   Reagent A: 0.2% (w/v) $AgNO_3$, 0.2% (w/v) NH_4NO_3, 1% (w/v)tungstosilicic acid, and 0.3% (v/v) formaldehyde (from a 37% stock solution in water) in distilled water. Store in the dark at room temperature.

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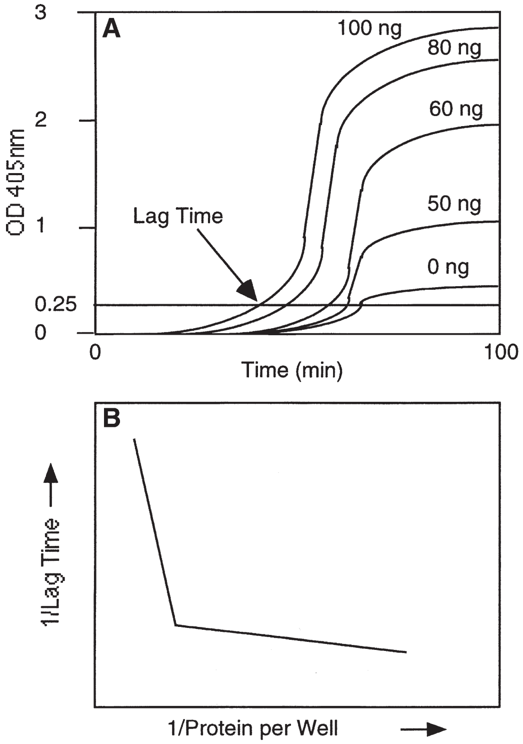

Fig. 1. Representative p\lots of standard curve data. **(A)** Raw data for varying standard protein mass per well. **(B)** Double-reciprocal plot of standard curve.

Reagent B: 5% (w/v) Na_2CO_3 in distilled water.

3. A microtiter plate reader is required (e.g., EIA Autoreader, model EL310, Biotek Instruments, Burlington, VT).
4. Cellulose paper or TLC plates.

3. Methods

3.1. Microtiter Plate Assay

1. Adsorb the protein of interest to duplicate microtiter plates (*see* **Note 1**). Wash the microtiter plate profusely with distilled water after the adsorption (*see* **Note 2**). One of the microtiter plates is used for the kinetic silver staining assay and the other is for quantitative ELISA or binding experiments. The microtiter plate for the kinetic silver staining assay contains protein adsorbed to only a few of the wells (e.g., 4–16 wells).
2. Prepare a known concentration of the standard protein in distilled water (e.g., by dialysis) for use in a standard curve (*see* **Notes 2** and **3**).
3. Apply varying concentrations (e.g., 50–1000 ng/mL) of the standard protein to the blank wells on the microtiter plate at the same volume (50–200 µL) that was used

to adsorb protein in **step 1**. Cover the microtiter plate with a tissue to avoid dust from settling in the wells and allow the protein to air dry for several days (*see* **Note 4**). Do not wash the plate after this step!

4. Mix equal volumes of reagents A and B immediately before use. A fixed volume (e.g., 100 µL) of the mixture is added quickly to the wells on the microtiter plate that were adsorbed with protein in **step 1, step 3,** and to blank control wells. The time for the addition of reagent to each well should be noted as time zero. All wells are filled in less than 10 min from the time of mixing reagents A and B; otherwise, excessive silver development in the reagent may cause a high background.
5. Read optical densities of each well with a microtiter plate reader at 405 nm (*see* **Note 5**). The time elapsed from time zero for each well to reach 0.25 OD (lag time) is noted as the lag time.
6. The lag time is plotted against the mass per well of adsorbed protein in the standard curve (**step 3**), which typically yields an inverse sigmoidal shape curve. Comparison of the lag times of the sample wells to the standard curve allows the determination of the total mass of protein in the sample well. The standard curve may be linearized to a sharply biphasic shape curve by plotting 1/lag time vs 1/protein per well (*see* **Fig. 1**).

3.2. Cellulose Assay

1. Mix equal volumes of reagents A and B immediately before use.
2. Stain cellulose (*see* **Note 6**) with adsorbed proteins by immersion in the mixed reagents for at least 1 h.

4. Notes

1. The wells on the edge of microtiter plates should be avoided for quantitative measurements because they tend to yield less accurate numbers.
2. Washing of microtiter plates is essential, as residual buffer reagents may interfere with silver staining (*see* **Table 1**). The washing is performed by gently dipping in beakers of deionized water. Vigorous washing was avoided for fear of losing adsorbed protein.
3. Kinetic silver staining shows little protein-to-protein variation (<30% over six tested proteins); it is possible to estimate the total amount of adsorbed protein or mixture of proteins using a standard (e.g., bovine albumin) that can be easily dissolved in water and measured for concentration.
4. Kinetic silver staining detects proteins bound to polystyrene and apparently not protein in solution; thus the quantitative adsorption of the standard protein in distilled water (to avoid interference, *see* **Note 2**) to polystyrene by drying is necessary. As the binding capacity of polystyrene is exceeded (typically about 100–200 ng/well, but depends upon both the microtiter plate and the protein of interest), kinetic silver staining does not detect further increases in protein mass per well.

Table 1
Compounds Known to Interfere with Kinetic Silver Staining

Compounds	Concentration in the staining reagent
TCA	>0.01 m*M*
Glucose	>1 m*M*
β-Mercaptoethanol	>0.1 m*M*
DTT	>0.01 m*M*
KOH	>0.01 m*M*
EDTA	>1 m*M*
Phosphate	>0.01 m*M*
SDS	>1 m*M*
Triton X-100	>0.0005%
Ammonium sulfate	>1 m*M*
Urea	>1 m*M*
Imidazole	>0.01 m*M*
Tris	>1 m*M*
NaCl	>0.01 m*M*
KCl	>0.01 m*M*
Guanidine HCl	>0.01 m*M*

5. Kinetic silver staining is based on measurements of light scattering. Thus, other wavelengths may be used, but the corresponding optical density reading will be different and may require optimization.
6. The cellulose may require washing to remove solvents or buffers, if present prior to staining.

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9

Quantitation of Cellular Proteins by Flow Cytometry

**Thomas D. Friedrich, F. Andrew Ray, Ralph L. Smith,
and John M. Lehman**

1. Introduction

Quantitation of specific proteins in complex mixtures is simplified by the use of antibodies directed against the protein of interest. If the specific protein is differentially expressed within a population of cells, quantitation of the protein in cell lysates by immunoblotting will provide an average quantity of the protein per cell. As a result, when lysates of different cell populations are compared, large changes in the amount of a protein in a small percentage of cells cannot be distinguished from small changes in the amount of a protein in a large percentage of cells. Flow cytometric analysis solves this problem by providing a means to measure the amount of a specific protein within each individual cell in a population. A fluorescently labeled probe, usually an antibody, is required for detection of the protein. A single cell suspension is reacted with an antibody and passed through a flow cytometer, which focuses the cell suspension into a stream that intersects a laser beam. As each cell passes through the beam, the fluorescent probe in each cell is excited and photomultiplier tubes register the degree of fluorescence ***(1)***.

Multivariate analysis, the simultaneous measurement of multiple parameters, is a powerful feature of flow cytometry. When determining the quantity of a specific protein in a cell, the quantity of other molecules in the same cell can also be measured by using additional fluors specifically targeted to other molecules. Examples of second molecules measured simultaneously include DNA ***(2–4)*** or additional proteins ***(5)***. In addition, measurement of light scatter can provide information about cell size and granularity. Most flow cytometers are capable of measuring six colors plus light scatter, but specialized flow cytometers that are capable of measuring as many as eighteen colors are now available.

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In this chapter we will describe techniques used to quantitate Simian Virus 40 (SV40) large T antigen (T Ag) in infected cells. By staining infected cells with antibodies against T Ag and with the DNA-binding dye propidium iodide it is possible to measure the amount of T Ag as a function of DNA content.

2. Materials

2.1 Cells and Antibodies

The CV-1 line of African Green monkey kidney cells was obtained from the American Type Culture Collection (ATTC, CCL-70). Confluent cultures of CV-1 were infected with Simian Virus 40 strain RH-911 at 100 plaque forming units per cell.

Pab101 is a monoclonal antibody specific to the carboxy-terminus of SV40 T Ag. Hybridoma cells producing Pab101 were obtained from ATCC (#TIB-117). Alexa conjugated secondary antibodies were obtained from Invitrogen.

2.2 Fixation and Staining

1. Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS): dissolve 8.0g NaCl, 0.2g KCl, 1.2g Na_2HPO_4 and 0.2g KH_2PO_4 in 1 liter of distilled water (dH_2O). Adjust the pH to 7.4, sterilize by passing through a 0.2 µm filter and store at room temperature.
2. Trypsin-EDTA: dilute 10X trypsin/EDTA (Invitrogen) in PBS to give final concentrations of 0.05% trypsin, 0.53 mM EDTA. Filter-sterilize and store at 4°C.
3. Methanol. Store at −20°C.
4. Wash solution (WS): heat inactivate 100 ml of normal goat serum (Invitrogen) at 56°C for 1 hour. Mix with 900 ml PBS, 20 µl Triton X-100 and 1.0g sodium azide. Filter-sterilize and store at 4°C (see **Note 1**).
5. Propidium iodide (PI): dissolve 10.0 mg PI (Calbiochem) in 100 ml PBS. Add 20 µl Triton X-100 and 0.1 g sodium azide. Protect solution from light and store at 4°C.
6. Ribonuclease: dissolve 100 mg of RNAase A (Sigma) in 100 ml PBS. Boil for 1 hour. Add 20 µl Triton X-100 and 0.1g sodium azide. Store at 4°C.

3. Methods

3.1 Fixation

1. Remove the culture medium and rinse the cells with PBS. If floating and/or mitotic cells are of interest, medium and wash fractions should be saved, pelleted and pooled with attached cells after trypsinization.
2. Prewarm trypsin/EDTA to 37°C and add 1 ml per 60 mm dish. (Adjust proportionally for larger culture areas). Tilt the plate to thoroughly distribute solution and then remove.
3. Once the cells have detached (see **Note 2**), add 1 ml of WS to the plate and transfer the cells to a 1.5 ml microcentrifuge tube. Remove a small volume for the determination of cell number (see **Note 3**). Centrifuge remaining cells at 4000 × g for 15 sec.
4. Discard supernatant, resuspend the pellet in 1 ml PBS at 4°C and centrifuge as in **step 3.**

5. Discard the supernatant and thoroughly resuspend cell pellet in 0.1 ml cold PBS.
6. Immediately add 0.9 ml methanol (−20°C) (see **Note 4**), mix and store at −20°C (see **Note 5**).

3.2 Titration of antibodies

It is important that the primary antibody (see **Note 6**) is present in excess to ensure quantitative measurement of the specific protein (see **Note 7**). However, excessive antibody can lead to increased background. In order to determine the appropriate antibody concentration, stain parallel samples of cells with serial dilutions of antibody. If available, similar cells that don't express the protein of interest are used for negative controls. Stain such cells in the same way as the experimental cells.

3.3 Staining

1. Transfer 1.5×10^6 cells to a 1.5 ml microcentrifuge tube and centrifuge at 4000 × g for 15 seconds (see **Note 8**).
2. Discard the supernatant and resuspend the cell pellet in 1 ml PBS at 4°C. Repeat centrifugation step.
3. Discard PBS and add 0.5 ml of diluted primary antibody. Mix gently.
4. Incubate with gentle agitation (see **Note 9**).
5. Pellet at 4000 × g for 15 sec and discard supernatant.
6. Resuspend the pellet in 0.5 ml WS and repeat **step 5.**
7. Resuspend the pellet in 0.5 ml of diluted fluorescently-labeled secondary antibody (see **Note 10**). Mix gently.
8. Incubate at 37°C for 2 h with gentle mixing; minimize exposure to light.
9. Add 0.5 ml RNAase and mix gently.
10. Incubate at 37°C for 30 min.
11. Add 0.5 ml PI, bringing the total volume to 1ml. Mix gently.
12. Filter each sample through a 53 µm mesh nylon grid (Nitex HC3-53, Tetko Inc.) (see **Note 11**).

3.4 Flow Cytometry and analysis

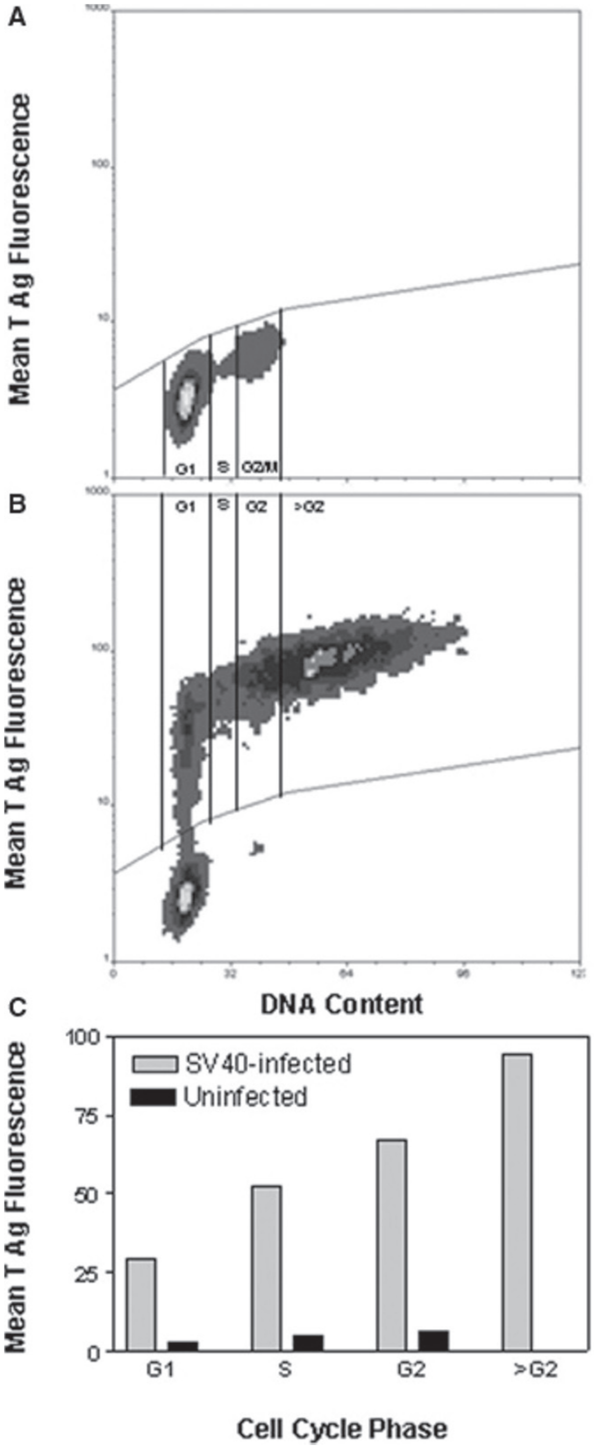
Most commercially available flow cytometers are capable of multiparameter analysis (see **Note 12**). Using 20 mW of power tuned to 488 nm, minimize the coefficient of variation (CV) for the green photomultiplier (see **Note 13**) to <2.0% by aligning the instrument with 2.0 µm fluorescent microspheres (Polysciences Inc.). Minimize the CV for red fluorescence (see **Note 14**) to <6.0% with PI-stained lymphocytes (see **Note 15**).

Set data acquisition to trigger on a photomultiplier collecting unfiltered 90° light scatter. Set gates to collect data representing single cells, and to eliminate data representing cell debris and cell aggregates. Compare red fluorescence to light scatter and set gate 1 to eliminate subcellular debris (low red fluorescence) and clumped cells (excessive light scatter). Then compare the peak height and area of red fluorescence and eliminate doublets (off axis owing to biphasic peak height). Collect and display red fluorescence vs green fluorescence. Collect data from at least 10,000 cells. Include appropriate control samples, save data and analyze. Representative data are shown in **Fig. 1**. For absolute quantitation (number of molecules of the specific protein per cell) a separate populations of cells with a known absolute quantities of the antigen can be used as standards (see **Note 16**).

4. Notes

1. Sodium azide is highly toxic. Consult the MSDS for proper handling instructions.
2. It is best to monitor cell detachment microscopically. Optimize cell treatment for the generation of a single cell suspension.
3. Each sample should contain 1–1.5 × 10^6 cells, because cells will be lost during fixation and staining. Use polypropylene tubes and minimize tube size to reduce cell loss.
4. The cell concentration can be adjusted at the time of fixation by adding the appropriate amounts of PBS (10%) and then methanol (90%). In some cases methanol fixation may result in loss of the protein antigen. Since the method of fixation can dramatically alter the results it may be necessary to test different fixation protocols. For example, fixation by 0.5% formal-

Fig. 1. Differential T antigen expression in SV40 infected monkey kidney cells. Cells in **A** and **B** were fixed and stained with propidium iodide (PI) and anti-T Ag as described in **Subheading 3.** PI staining (DNA content) is expressed on a linear scale and anti- T Ag fluorescence on a log scale. **(A)** An uninfected culture of confluent CV-1 cells. The cell cycle distribution, as determined by PI staining was: 80% G1 phase, 5% S phase, 12% G2/M phase and 3% >G2 phase. **(B)** Confluent CV-1 cells infected with SV40 at a multiplicity of infection of 100 plaque forming units per cell. Cells were trypsinized and fixed at 48 hours post infection. The *sloped horizontal line* in each panel was set to divide T Ag positive from T Ag negative cells. *Vertical lines* indicate gates that were set on the basis of DNA content to discriminate cells within the different phases of the cell cycle. The cell cycle distribution of the T Ag expressing cells was: 27% G1 phase, 9% S phase, 14% G2/M phase and 50% >G2 phase. Levels of T Ag expression in G1 phase cells cover a 10-fold range, but only cells expressing higher amounts of T Ag enter S phase. >G2 phase represents a virus-regulated override of normal cell cycle events. The cells in >G2 phase are infected cells that are replicating viral DNA and re-replicating cellular DNA **(7).** The G1 phase cells with fluorescence levels similar to the background levels of the uninfected cells are believed to be an uninfected subpopulation that conveniently serves as an internal control in this system. **(C)** The relative quantities of T Ag within each cell cycle gate, as determined by anti T Ag staining. The bar graph shows the average quantity of T Ag per cell in the T Ag expressing population.


A
Mean T Ag Fluorescence
1000
100
10
1
G1
S
G2/M
B
Mean T Ag Fluorescence
1000
100
10
1
G1
S
G2
>G2
0
32
64
96
DNA Content
C
Mean T Ag Fluorescence
100
75
50
25
0
SV40-infected
Uninfected
G1
S
G2
>G2
Cell Cycle Phase

dehyde followed by methanol has been used to retain antigens lost with methanol fixation alone ***(6)***.

5. Fixed cells have been stored at –20°C for >1 year with negligible loss of T Ag. However stability during storage must be evaluated for each antigen.
6. Monoclonal antibodies or affinity purified polyclonal antibodies are preferred. Polyclonal antisera may have additional antibodies of unknown specificity that will increase background staining.
7. Antibody binding is actually a quantitative measure of the epitope recognized by the antibody and not necessarily the total amount of the protein antigen. If the epitope is masked through protein conformation/association or as an artifact of fixation, the epitope may not be detected.
8. Use of a swinging bucket microcentrifuge reduces cell loss.
9. Adjust time and temperature to maximize the signal over background. Incubation times generally range from 30 minutes to 2 hours at 37°C or overnight at 4°C. Gentle rocking is recommended.
10. Primary antibodies that are directly conjugated to fluors are commercially available. In addition, kits that allow conjugation of Alexa fluors to antibodies can be obtained from Invitrogen. If particularly weak signals are encountered, the signal can be amplified by additional layers of fluorescently-tagged antibody. For example, cells stained with an FITC-labeled primary or secondary antibody can be reacted with Alexa 488-labeled rabbit anti-fluorescein followed by Alexa 488-labeled goat anti-rabbit IgG.
11. Filtering of samples removes cell aggregates that may clog the tubing in the flow cytometer. If samples are stored and then reanalyzed, filtering should be repeated.
12. We have used a Cytofluorograph Model 50 H-H with an air-cooled argon laser (model 532, Omnichrome). The Cyclops analysis program (Cytomation) was used for data analysis. More recently a BD FACScan has been utilized with similar settings and filters and identical results were obtained.
13. Use a 535 nm band pass filter for this photomultiplier.
14. Use a 640 nm long pass filter for this photomultiplier.
15. PI stained lymphocytes are prepared by first making a stock of spleen cells from a healthy mouse. Spleens are removed and minced with fine surgical scissors in PBS. The cells in the PBS supernatant are removed and placed in a centrifuge tube. Remaining spleen fragments are minced in PBS until few cells are evident in the supernatants. Cells in the pooled supernatants are pelleted by centrifugation and washed 2X with PBS. The pellet is resuspended in 1ml of PBS, mixed with 9ml of –20° C methanol and stored at –20° C. Prior to each run, wash and stain 1×10^6 cells with PI/RNAse only.

16. Values obtained from flow cytometric analysis do not provide an absolute quantitation (numbers per cell) of an epitope, but rather represent relative quantities of the epitope within cells in a population. A common method of determining the number of epitopes per cell is to compare the fluorescent intensity of the cell to standard beads that have a known number of primary antibody molecules per bead. The same fluorescently-labeled antibody that is used to stain cells is also reacted with the standard beads under the same conditions. Alternatively, if the number of fluorochrome molecules per antibody molecule is known, a comparison of a cell's fluorescent intensity to that of standard beads with a known number of bound fluorochrome molecules is possible. Both types of beads are commercially available.

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10

Quantitation of Cellular Proteins by Laser Scanning Cytometry

Thomas D. Friedrich, Ralph L. Smith, and John M. Lehman

1. Introduction

Flow Cytometry (FC) is a popular method for the simultaneous quantitation of multiple specific proteins and other molecules in each cell of a single cell suspension ***(1)***. Laser Scanning Cytometry (LSC) is a closely related technology that provides many of the benefits of FC and offers additional features that allow morphological and biochemical analysis of a cell population ***(2,3)***. In both methods, quantitation of endogenous cellular proteins generally requires antibodies against the protein of interest. These antibodies must be either directly labeled with a fluorochrome or detected by a fluorescently-labeled secondary antibody. Using fluorescently-labeled antibodies in combination with fluorescent dyes that quantitatively bind to DNA, both FC and LSC have been valuable in measuring the quantities of specific proteins in relation to cell cycle position ***(4)***. In contrast to FC, where a single cell suspension is passed through a fixed-position laser beam, LSC analyzes cells fixed to a surface such as a microscope slide or cover slip. Since the position of the cell on the slide is part of the recorded data, it is possible to visualize the cell after the initial recording event or to subsequently stain the cell with another fluorescent probe and make a second measurement. LSC also allows analysis of cells following biochemical treatments that are incompatible with analysis by FC. In this chapter we describe the value of LSC in analyzing chromatin-associated proteins. Specifically, we describe an in situ fractionation procedure that allows measurement of total and chromatin-associated MCM2, a DNA helicase subunit, as a function of cell cycle position.

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2. Materials

2.1. Cells and Antibodies

The CV-1 line of African Green monkey kidney cells was obtained from the American Type Culture Collection (ATCC, CL-70). Cells were maintained in MEM/ 5% fetal bovine serum. BM28 (#610701), a monoclonal antibody that specifically recognizes MCM2 was obtained from BD Biosciences. Alexa-488 conjugated goat anti-mouse IgG (#A-11001) was obtained from Invitrogen.

2.2. Fixation and Staining

1. Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS): Dissolve 8.0g NaCl, 0.2g KCl, 1.2g Na_2HPO_4 and 0.2g KH_2PO_4 in 1 liter of distilled water (dH_2O). Adjust the pH to 7.4, sterilize by passing through a 0.2 µm filter and store at room temperature.
2. Wash solution (WS): heat inactivate 100 ml of normal goat serum (Invitrogen) at 56°C for 1 hour. Mix with 900 ml PBS, 20 µl Triton X-100 and 1.0g sodium azide. Filter-sterilize and store at 4°C (see **Note 1**).
3. Propidium iodide (PI): dissolve 10.0 mg PI (Calbiochem) in 100 ml PBS. Add 20 µl Triton X-100 and 0.1 g sodium azide. Protect solution from light and store at 4°C.
4. Ribonuclease: dissolve 100 mg of RNAase A (Sigma) in 100 ml PBS. Boil for 1 hour. Add 20 µl Triton X-100 and 0.1 g sodium azide. Store at 4°C.

2.3. Cell Extraction

1. 2X CSK buffer (200 ml): bring 0.6 g PIPES, 20.5 g sucrose, (see **Note 2**), 4 ml 5 M NaCl and 0.6 ml of 1M $MgCl_2$ to total volume of 190 ml with H_2O. The solution will clear, with stirring, while adjusting to pH 7.0 with 10N NaOH. Add H_2O to a final volume of 200 ml. (1X CSK= 10 mM PIPES pH7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$).
2. 100X protease inhibitor cocktail: dissolve 2.5 mg leupeptin, 2.5 mg aprotinin, 15.0 mg benzamidine and 1.0 mg trypsin inhibitor (all protease inhibitors were from Sigma) in 1.0 ml H_20. Store in 0.1 ml aliquots at –20° C.
3. CSK/0.5% Triton X-100. For 5 ml, combine 2.5 ml 2X CSK buffer, 2.15 ml H_20, 0.25 ml 10% Triton X-100, 0.05 ml 100 mM PMSF and 0.05 ml 100X protease inhibitor cocktail.

3. Methods

3.1. CSK Extraction and Fixation

This procedure was adapted from reports describing the use of a selective extraction procedure to demonstrate the release of MCM proteins from chromatin in S phase cells ***(6,7,8)***.

1. Cells can be grown in culture slides or on glass cover slips placed in tissue culture plates. For the purpose of this study, CV-1 cells were grown on 12 mm circular glass cover slips in 60 mm plates.
2. At time of harvest, aspirate medium and wash plate with 5 ml PBS.

3. Remove cover slips with fine-tipped forceps and place cell-side up on a parafilm covered glass plate in a humidified chamber. (see **Note 3**)
4. Gently place 150 µl CSK buffer on each cover slip (see **Note 4**).
5. Aspirate CSK and replace with 100-150 µl CSK/0.5% Triton X-100; keep in humidified chamber for 5 min.
6. Aspirate CSK/0.5% Triton X-100 and gently wash 3X with CSK buffer (see **Note 5**).
7. Fix immediately by transferring cover slips to Alumina staining racks (Thomas Scientific) submerged in methanol at −20°C (see **Note 6**).
8. Allow cover slips to air dry; either store at −20°C or continue with antibody staining.

3.2. Titration of Antibodies

Quantitative measurement of a specific protein requires that antibody be present in excess. Yet, the background of nonspecific antibody staining should be kept to a minimum. To determine the appropriate antibody concentration, stain parallel cover slips with serial dilutions of antibody. If similar cells not expressing the protein of interest are available, they can be stained at the same dilutions to determine levels of nonspecific staining. Background staining due to nonspecific binding by the primary antibody can also be measured in cells stained with nonspecific antibody of the same isotype. Background staining due to the fluorescent secondary antibody can be measured in cells stained with the secondary antibody only.

3.3. Protein and DNA Staining

1. Rehydrate dried cover slips by placing cell-side up on a parafilm covered glass plate in a humidified chamber and covering with 150 µl wash solution.
2. Remove WS and layer 20-50 µl of primary antibody diluted in WS onto each cover slip. Incubate 30 min at 37°C (see **Note 7**).
3. Rinse cover slips 3X by gentle addition and aspiration of 150 µl wash solution.
4. Layer 20-50 µl of fluorescently labeled secondary antibody onto each cover slip and incubate 30 min at 37°C (see **Note 8**).
5. Rinse cover slips 5X by gentle addition and aspiration of 150 µl wash solution.
6. Combine equal volumes of PI solution and RNAase solution. Incubate each cover slip with 20-50 µl of the mixture for 30 min at room temperature.
7. Briefly rinse cover slip by dipping in H_20 and drain by touching edge of cover slip to a Kimwipe. Prepare mounting medium by mixing 90% glycerol/ 10% PBS and then diluting 1:1 with PI solution. Apply 5-10 µl of mounting media to a glass microscope slide and mount cover slip cell side down (see **Note 9**).

3.4. Laser Scanning Cytometry and Data Analysis

The data was acquired with an iCys Research Imaging Cytometer manufactured by CompuCyte, Cambridge, MA, which is capable of scanning micro plates, slides, and other carriers. This microscope-based instrument uses three lasers (488 nm argon, 633 nm helium neon, and 405 nm violet diode), dichroic mirrors, optical filters (650 LP for CY5, 580/30 for PE, 530/30 for FITC, and 463/39 for DAPI), and photomultipliers (PMTs). Software utilized was the iGeneration Cytometric Analysis Software Version 3.2.5. Instrument settings were 5 mW laser power (488nm argon) with the detector voltages set at 35 V green channel with a 530/30 optical filter and 33 V long red channel with a 650 LP optical filter. For reanalysis, the contour primary minimum area of 20 μm^2 was set to exclude cell fragments and a maximum area of 340 μm^2 to allow doublet discrimination. The instrument will acquire the data and record the location of each cell. Therefore, after the data is acquired, stored and analyzed, the cells may be viewed in a gallery for their morphology and surrounding cells. Cells can be restained with other fluorochromes/antibodies if required.

The procedure detailed above was used to examine the expression and nuclear association of the DNA helicase subunit, MCM2, in proliferating monkey kidney cells. **Figure 1A** shows MCM2 (*y* axis) vs DNA staining (*x* axis) of cells directly fixed in methanol. The greatest range in MCM2 expression is observed in G1 phase cells. Cells in S and G2 phases uniformly express higher levels of MCM2. **Panel B** shows cells that were extracted with CSK/0.5% Triton X-100 to remove soluble proteins prior to methanol fixation. A striking characteristic of extracted S phase cells is the decrease in MCM2 as cells increase in DNA content, whereas the directly fixed cells have consistently high levels of MCM2 throughout S phase. The conclusion from this analysis is that although the level of nuclear MCM2 remains constant throughout S phase, the increase in DNA content during S phase directly correlates with release of chromatin-bound MCM2. The release of MCM proteins as a consequence of replication origin firing has been described previously in biochemical and immunofluorescent microscopy studies ***(6-8)***. However, the multiparameter capabilities of LSC provide the advantage of simultaneous quantitation of MCM2 protein and DNA ***(5)***.

Another powerful feature of LSC, which is not possible with FC, is the ability to visualize laser scan images of each event assembled in an image gallery. This is particularly useful in further defining specific events and eliminating undesirable events from the scattergrams. An example is seen in panel B where there appear to be subpopulations of extracted cells that retain MCM2 in G2/M phase and higher DNA contents. Examination of an image gallery revealed that these events were not single cells, but doublets and clusters of multiple nuclei. The acquired data (**panels A and B**), were reanalyzed to eliminate doublets and large nuclear clusters based on their area (see **Note 10**). Morphological identification of the cells from image galleries of the G2/M regions of **panels B** and **D** is presented is presented in **panels E** and **F**, respectively. Only nuclei are apparent due to visualization of the PI stain. Cells viewed in **panel E** prior to exclusion of events greater than 340 μm^2 exhibit numerous doublets and multiple clusters, while the cells viewed following exclusion (**panel F**) are single cells. The scattergrams in **panels C** and **D** exclude events greater than 340 μm^2 from **panels A**

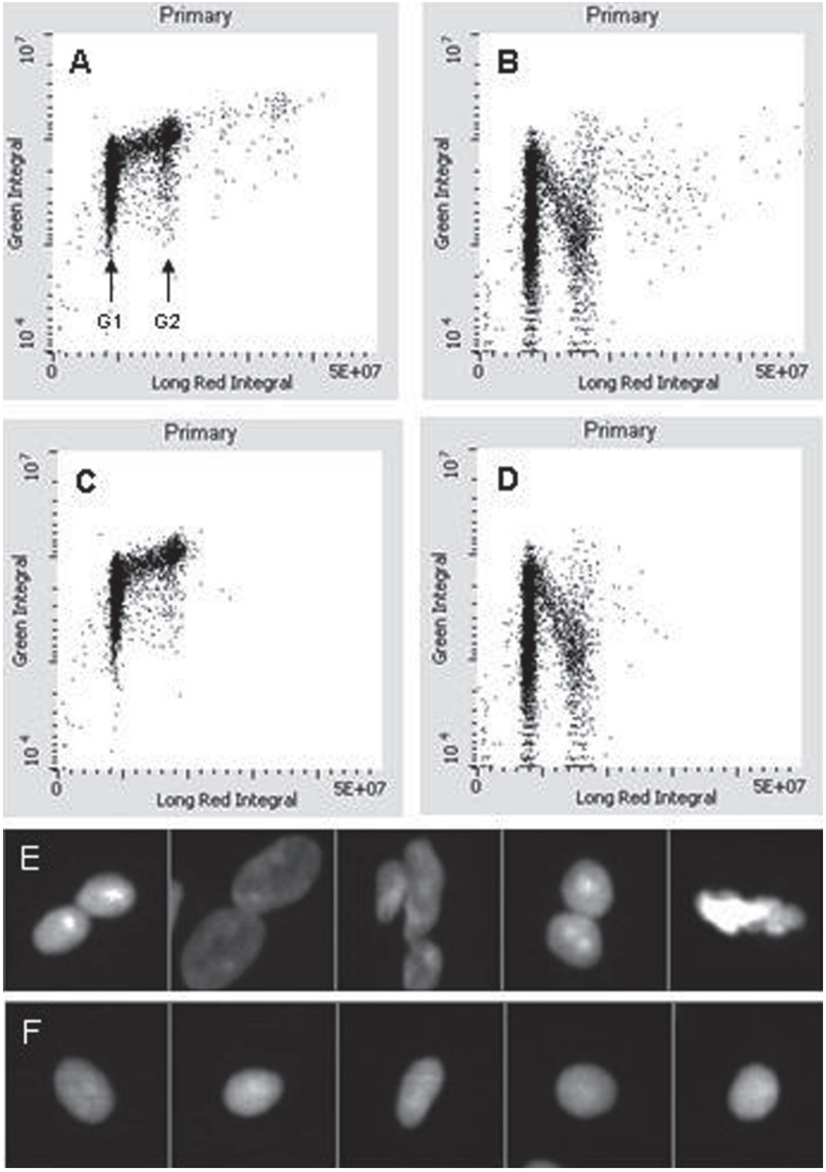

Fig. 1. Growing CV-1 monkey kidney cells stained for MCM2 (*y* axis) and DNA content (*x* axis). **(A)** Cells were directly fixed in methanol and then stained for MCM2 and DNA. **(B)** Parallel cover slips were extracted with CSK/0.5% Triton X-100 prior to fixation and staining. To eliminate doublet and larger nuclear clusters from the scattergrams, PI-stained events with an area greater than 340 µm^2 were subtracted from the scattergrams A and B to generate the distributions in **(C)** directly fixed and **(D)** extracted. **(E)** A gallery of nuclei excluded by elimination of events greater than 340 µm^2 from the G2/M phase region. **(F)** A gallery of nuclei with areas between 20 and 340 µm^2 from the G2/M phase region.

and **B,** respectively, and significantly reduce two artifactual results. First, cells that appear to have DNA contents greater than that of a G2/M phase cell are nearly eliminated. Second, a cell subpopulation that appears to retain MCM2 binding in G2/M and cells of higher DNA content is markedly decreased.

The ability to perform *in situ* extractions brings elements of biochemical fractionation to the field of quantitative cytometry. Additional fractionation procedures or other biochemical manipulations should be feasible as long as primary cell morphology and the epitope being examined are not destroyed. In addition, the capacity of this instrumentation to reanalyze data provides a powerful tool to determine and characterize multiple parameters of the collected data.

4. Notes

1. Sodium azide is highly toxic. Consult the MSDS for proper handling instructions.
2. Use a molecular biology-grade sucrose, such as Sigma S0389.
3. A simple humidified chamber can be made from a plastic or glass 150 mm Petri dish. Filter paper or paper towel is cut to fit inside the lid and moistened with H_20. A glass plate such as an 8 × 10 cm minigel plate is wrapped in parafilm and placed on the moistened paper. The base of the dish is used as the lid.
4. An alternative to individual treatment of cover slips is to leave cover slips in the tissue culture plate and treat with larger volumes of extraction and wash buffers.
5. Cells treated with CSK/0.5% Triton X-100 are more susceptible to detachment from the cover slip. Extra care must be taken in adding and removing buffer from the cover slip.
6. 70% acetone/30% methanol has also been successfully used for fixation of MCM2 (5). Appropriate fixation conditions will need to be optimized for each epitope.
7. Longer periods of incubation (1-2 hours) at room temp may also be used and should be optimized for each epitope.
8. To protect fluorescently labeled antibody from light, use aluminum foil to wrap the base and lid of the humidified chamber, described in **Note 3.**
9. If more permanent mounting is required, the cover slips can be mounted in ProLong (Invitrogen) supplemented with PI.
10. Doublets and clusters of multiple nuclei can be excluded based on the area (measured in square microns) of each event. For example, two G1 phase nuclei in close proximity may be recorded as a single event. Although the DNA content of the two G1 phase nuclei will be recorded as equivalent to a G2 phase nucleus, the area of the two G1 nuclei will be greater than that of the G2 nucleus. In this case, doublets and larger nuclear clusters

were eliminated by excluding events with an area greater than 340 μm^2. The optimal exclusion threshold should be determined empirically for each experimental group.

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PART II

ELECTROPHORESIS OF PROTEINS AND PEPTIDES AND DETECTION IN GELS

11

Protein Solubility in Two-Dimensional Electrophoresis

Basic Principles and Issues

Thierry Rabilloud

1. Introduction

The solubilization process for two-dimensional (2-D) electrophoresis has to achieve four parallel goals:

1. Breaking macromolecular interactions in order to yield separate polypeptide chains. This includes denaturing the proteins to break noncovalent interactions, breaking disulfide bonds, and disrupting noncovalent interactions between proteins and nonproteinaceous compounds such as lipids or nucleic acids.
2. Preventing any artefactual modification of the polypeptides in the solubilization medium. Ideally, the perfect solubilization medium should freeze all the extracted polypeptides in their exact state prior to solubilization, both in terms of amino acid composition and in terms of posttranslational modifications. This means that all the enzymes able to modify the proteins must be quickly and irreversibly inactivated. Such enzymes include of course proteases, which are the most difficult to inactivate, but also phosphatases, glycosidases, and so forth. In parallel, the solubilization protocol should not expose the polypeptides to conditions in which chemical modifications (e.g., deamidation of Asn and Gln, cleavage of Asp-Pro bonds) may occur.
3. Allowing the easy removal of substances that may interfere with 2-D electrophoresis. In 2-D electrophoresis, proteins are the analytes. Thus, anything in the cell but proteins can be considered as an interfering substance. Some cellular compounds (e.g., coenzymes, hormones) are so dilute they go unnoticed. Other compounds (e.g., simple nonreducing sugars) do not interact with proteins or do not interfere with the electrophoretic process. However, many compounds bind to proteins and/or interfere with 2-D electrophoresis and must be eliminated prior to electrophoresis if their amount exceeds a critical interference threshold. Such compounds mainly include salts, lipids, polysaccharides (including cell walls), and nucleic acids.

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4. Keeping proteins in solution during the 2-D electrophoresis process. Although solubilization *stricto sensu* stops at the point where the sample is loaded onto the first dimension gel, its scope can be extended to the 2-D process *per se*, as proteins must be kept soluble till the end of the second dimension. Generally speaking, the second dimension is an SDS gel, and very few problems are encountered once the proteins have entered the SDS-polyacrylamide gel electrophoresis (SDS PAGE) gel. The one main problem is overloading of the major proteins when micropreparative 2-D electrophoresis is carried out, and nothing but scaling-up the SDS gel (its thickness and its other dimensions) can counteract overloading an SDS gel. However, severe problems can be encountered in the isoelectric fusing (IEF) step. They arise from the fact that IEF must be carried out in low ionic strength conditions and with no manipulation of the polypeptide charge. IEF conditions give problems at three stages:
    a. During the initial solubilization of the sample, important interactions between proteins of widely different pI and/or between proteins and interfering compounds (e.g., nucleic acids) may happen. This yields poor solubilization of some components.
    b. During the entry of the sample in the focusing gel, there is a stacking effect due to the transition between a liquid phase and a gel phase with a higher friction coefficient. This stacking increases the concentration of proteins and may give rise to precipitation events.
    c. At, or very close to, the isoelectric point, the solubility of the proteins comes to a minimum. This can be explained by the fact that the net charge comes close to zero, with a concomitant reduction of the electrostatic repulsion between polypeptides. This can also result in protein precipitation or adsorption to the IEF matrix.

Apart from breaking molecular interactions and solubility in the 2-D gel, which are common to all samples, the solubilization problems encountered will greatly vary from a sample type to another because of wide differences in the amount and nature of interfering substances and/or spurious activities (e.g., proteases). The aim of this outline chapter is not to give detailed protocols for various sample types, and the reader should refer to the chapters of this book dedicated to the type of sample of interest. I would rather like to concentrate on the solubilization rationale and to describe nonstandard approaches to solubilization problems. More detailed review on solubilization of proteins for electrophoretic analyses can be found elsewhere ***(1,2)***.

2. Rationale of Solubilization-Breaking Molecular Interactions

Apart from disulfide bridges, the main forces holding proteins together and allowing binding to other compounds are noncovalent interactions. Covalent bonds are encountered mainly between proteins and some coenzymes. The noncovalent interactions are mainly ionic bonds, hydrogen bonds, and "hydrophobic interactions." The basis for "hydrophobic interactions" is, in fact, the presence of water. In this very peculiar (hydrogen-bonded, highly polar) solvent, the exposure of nonpolar groups to the solvent is thermodynamically not favored compared to the grouping of these apolar groups together. Indeed, although the van der Waals forces give an equivalent contribution in both configurations, the other forces (mainly hydrogen bonds) are maximized in the latter configuration and disturbed in the former (solvent destruction). Thus, the energy balance in clearly in favor of the collapse of the apolar groups together ***(3)***. This explains why hexane and water are not miscible, and also that the lateral chain of apolar amino acids (L, V, I, F, W, Y) pack together and form the hydrophobic cores of the proteins ***(4)***. These hydrophobic interactions are also responsible for some protein-protein interactions and for the binding of lipids and other small apolar molecules to proteins.

The constraints for a good solubilization medium for 2-D electrophoresis are therefore to be able to break ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide bridges under conditions compatible with IEF (i.e., with very low amounts of salt or other charged compounds) (e.g., ionic detergents).

2.1. Disruption of Disulfide Bridges

Breaking of disulfide bridges is usually achieved by adding to the solubilization medium an excess of a thiol compound. Mercaptoethanol was used in the first 2-D protocols ***(5)***, but its use does have drawbacks. Indeed, a portion of the mercaptoethanol will ionize at basic pH, enter the basic part of the IEF gel, and ruin the pH gradient in its alkaline part because of its buffering power ***(6)***. Although its pK is approximately 8, dithiothreitol is much less prone to this drawback, as it is used at much lower concentrations (usually 50 m*M* instead of the 700 m*M* present in 5% mercaptoethanol). However, DTT is still not the perfect reducing agent. Some proteins of very high cysteine content or with cysteines of very high reactivity are not fully reduced by DTT. In these cases, phosphines are very often an effective answer. First, the reaction is stoichiometric, which allows in turn to use very low concentration of the reducing agent (a few m*M*). Second, these reagents are not as sensitive as thiols to dissolved oxygen. The most powerful compound is tributylphosphine, which was the first phosphine used for disulfide reduction in biochemistry ***(7)***. However, the reagent is volatile, toxic, has a rather unpleasant odor, and needs an organic solvent to make it water-miscible. In the first uses of the reagent, propanol was used as a carrier solvent at rather high concentrations (50%) ***(7)***. It was however found that DMSO or DMF are suitable carrier solvents, which enable the reduction of proteins by 2 m*M* tributylphosphine ***(8)***. All these drawbacks have disappeared with the introduction of a water-soluble phospine, tris (carboxyethyl) phosphine (available from Pierce), for which 1M aqueous stock solutions can be easily prepared and stored frozen in aliquots. The successful use of tributylphosphine in two-dimensional electrophoresis has been reported ***(9)***. However, the benefits over DTT do not seem obvious in many cases ***(10)***, although th use of phosphines allows in turn to use thiol-blocking agents (e.g., dithiodiethanol), resulting in iproved resolution of the basic proteins ***(11,12)***.

2.2. Disruption of Noncovalent Interactions

The perfect way to disrupt all types of noncovalent interactions would be the use of a charged compound that disrupts hydrophobic interactions by providing a hydrophobic environment. The hydrophobic residues of the proteins would be dispersed in that environment and not clustered together. This is just the description of SDS, and this explains why SDS has been often used in the first stages of solubilization ***(13–16)***. However, SDS is not compatible with IEF, and must be removed from the proteins during IEF.

The other way of breaking most noncovalent interactions is the use of a chaotrope. It must be kept in mind that all the noncovalent forces keeping molecules together must be taken into account with a comparative view on the solvent. This means that the final energy of interaction depends on the interaction *per se* and on its effects on the solvent. If the solvent parameters are changed (dielectric constant, hydrogen bond formation, polarizability, etc.), all the resulting energies of interaction will change. Chaotropes, which alter all the solvent parameters, exert profound effects on all types of interactions. For example, by changing the hydrogen bond structure of the solvent, chaotropes disrupt hydrogen bonds but also decrease the energy penalty for exposure of apolar groups and therefore favor the dispersion of hydrophobic molecules and the unfolding of the hydrophobic cores of a protein ***(1,17)***. Unfolding the proteins will also greatly decrease ionic bonds between proteins, which are very often not very numerous and highly dependent of the correct positioning of the residues. As the gross structure of proteins is driven by hydrogen bonds and hydrophobic interactions, chaotropes decrease dramatically ionic interactions both by altering the dielectric constant of the solvent and by denaturing the proteins, so that the residues will no longer positioned correctly.

Nonionic chaotropes, as those used in 2-D electrophoresis, however, are unable to disrupt ionic bonds when high charge densities are present (e.g., histones, nucleic acids) ***(18)***. In this case, it is often quite advantageous to modify the pH and to take advantage of the fact that the ionizable groups in proteins are weak acids and bases. For example, increasing the pH to 10 or 11 will induce most proteins to behave as anions, so that ionic interactions present at pH 7 or lower turn into electrostatic repulsion between the molecules, thereby promoting solubilization. The use of a high pH results therefore in dramatically improved solubilizations, with yields very close to what is obtained with SDS ***(19)***. The alkaline pH can be obtained either by addition of a few m*M* of potassium carbonate to the urea-detergent-ampholytes solution ***(19)***, or by the use of alkaline ampholytes ***(16)***, or by the use of a spermine-DTT buffer which allows better extraction of nuclear proteins ***(20)***.

For 2-D electrophoresis, the chaotrope of choice is urea. Although urea is less efficient than substituted ureas in breaking hydrophobic interactions ***(17)***, it is more efficient in breaking hydrogen bonds, so that its overall solubilization power is greater. It has been found that thiourea in addition to urea results in superior solubilization of proteins ***(21)*** and also in improved protein focusing. This may be explained by the fact that thiourea is a superior to urea for protein denaturation, being overwhelmed only by guanidine ***(22)***. However, the solubility of thiourea in water is not sufficient to draw full benefits from its denaturing power, so that it must be used in admixture with urea. However, denaturation by chaotropes induces the exposure of the totality of the proteins hydrophobic residues to the solvent. This increases in turn the potential for hydrophobic interactions, so that chaotropes alone are often not sufficient to quench completely the hydrophobic interactions, especially when lipids are present in the sample. This explains why detergents, which can be viewed as specialized agents for hydrophobic interactions, are almost always included in the urea-based solubilization mixtures for 2-D electrophoresis. Detergents act on hydrophobic interactions by providing a stable dispersion of a hydrophobic medium in the aqueous medium, through the presence of micelles for example. Therefore, the hydrophobic molecules (e.g., lipids) are no longer collapsed in the aqueous solvent but will disaggregate in the micelles, provided the amount of detergent is sufficient to ensure maximal dispersion of the hydrophobic molecules. Detergents have polar heads that are able to contract other types of noncovalent bonds (hydrogen bonds, salt bonds for charged heads, etc.). The action of detergents is the sum of the dispersive effect of the micelles on hydrophobic part of the molecules and the effect of their polar heads on the other types of bonds. This explains why various detergents show very variable effects varying from a weak and often incomplete delipidation (e.g., Tweens) to a very aggressive action where the exposure of the hydrophobic core in the detergent-containing solvent is no longer energetically unfavored and leads to denaturation (e.g., SDS).

Of course, detergents used for IEF must bear no net electrical charge, and only nonionic and zwitterionic detergents may be used. However, ionic detergents such as SDS may be used for the initial solubilization prior to isoelectric focusing to increase solubilization and facilitate the removal of interfering compounds. Low amounts of SDS can be tolerated in the subsequent IEF ***(13)*** provided that high concentrations of urea ***(23)*** and nonionic ***(13)*** or zwitterionic detergents ***(24)*** are present to ensure complete removal of the SDS from the proteins during IEF. Higher amounts of SDS must be removed prior to IEF, by precipitation ***(13)*** for example. It must therefore be kept in mind that SDS will only be useful for solubilization and for sample entry, but will not cure isoelectric precipitation problems.

The use of nonionic or zwitterionic detergents in the presence of urea presents some problems due to the presence of urea itself. In concentrated urea solutions, urea is not freely dispersed in water but can form organized channels (see ***[20]***). These channels can bind linear alkyl chains, but not branched or cyclic molecules, to form complexes of undefined stoichiometry called inclusion compounds. These complexes are much less soluble than the free solute, so that precipitation is often induced upon formation of the inclusion compounds, precipitation being stronger with increasing alkyl chain length and higher urea concentrations. Consequently, many nonionic or zwitterionic detergents with linear hydrophobic tails ***(24,25)*** and some ionic ones ***(28)*** cannot be used in the presence of high concentrations of urea. This limits the choice of detergents mainly to those with nonlinear alkyl tails (e.g., Tritons, Nonidet P40, CHAPS) or with short alkyl tails (e.g., octyl glucoside), which are unfortunately less efficient in quenching hydrophobic interactions. However, sulfobetaine detergents with long linear alkyl tails have received limited applications, as they require low concentrations of urea. However, good results have been obtained in certain cases for sparingly soluble proteins ***(27–29)***, although this type of protocol seems rather delicate owing to the need for a precise control of all parameters to prevent precipitation. Thus, protocols using detergents with a good urea compatibility are generally preferred, both because of their simpler use and because synergistic effects between the urea-thiourea chaotrope mixture and some detergents have been observed ***(32)***.

Apart from the problem of inclusion compounds, the most important problem linked with the use of urea is carbamylation. Urea in water exists in equilibrium with ammonium cyanate, the level of which increases with increasing temperature and pH ***(33)***. Cyanate can react with amines to yield substituted urea. In the case of proteins, this reaction takes place with the α-amino group of the N-terminus and the ε-amino groups of lysines. This reaction leads to artefactual charge heterogeneity, N-terminus blocking and adduct formation detectable in mass spectrometry. Carbamylation should therefore be completely avoided. This can be easily made with some simple precautions. The use of a pure grade of urea (p.a.) decreases the amount of cyanate present in the starting material. Avoidance of high temperatures (never heat urea-containing solutions above 37°C) considerably decreases cyanate formation. In the same trend, urea-containing solutions should be stored frozen (–20°C) to limit cyanate accumulation. Last, but not least, a cyanate scavenger (primary amine) should be added to urea-containing solutions. In the case of isoelectric focusing, carrier ampholytes are perfectly suited for this task. If these precautions are correctly taken, proteins seem to withstand long exposures to urea without carbamylation ***(34)***.

3. Methods

3.1 Solubility During IEF

Additional solubility problems often arise during the IEF at sample entry and solubility at the isoelectric point.

3.1.1 Solubility During Sample Entry

Sample entry is often quite critical. In most 2-D systems, sample entry in the IEF gel corresponds to a transition between a liquid phase (the sample) and a gel phase of higher friction coefficient. This induces a stacking of the proteins at the sample-gel boundary, which results in very high concentration of proteins at the application point. These concentrations may exceed the solubility threshold of some proteins, thereby inducing precipitation and sometimes clogging of the gel, with poor penetration of the bulk of proteins. Such a phenomenon is of course more prominent when high amounts of proteins are loaded onto the IEF gel. The sole simple but highly efficient remedy to this problem is to include the sample in the IEF gel. This process abolishes the liquid-gel transition and decreases the overall protein concentration, as the volume of the IEF gel is generally much higher than the one of the sample.

This process is however rather difficult for tube gels in carrier ampholyte-based IEF. The main difficulty arises from the fact that the thiol compounds used to reduce disulfide bonds during sample preparation are strong inhibitors of acrylamide polymerization, so that conventional samples cannot be used as such. Alkylation of cysteines and of the thiol reagent after reduction could be an answer, but many neutral alkylating agents (e.g., iodoacetamide, ethyl maleimide) also inhibit acrylamide polymerization. Owing to this situation, most workers describing inclusion of the sample within the IEF gel have worked with nonreduced samples ***(35,36)***. Although this presence of disulfide bridges is not optimal, inclusion of the sample within the gel has proven of great, but neglected, interest ***(35,36)***. It must however be pointed out that it is now possible to carry out acrylamide polymerization in an environment where disulfide bridges are reduced. The key is to use 2 m*M* tributylphosphine as the reducing agent in the sample and using tetramethylurea as a carrier solvent. This ensures total reduction of disulfides and is totally compatible with acrylamide polymerization with the standard Temed/persulfate initiator (T. Rabilloud, unpublished results). This modification should help the experimentators trying sample inclusion within the IEF gel when high amounts of proteins are to be separated by 2D.

The process of sample inclusion within the IEF gel is however much simpler for IPG gels. In this case, rehydration of the dried IPG gel in a solution containing the protein sample is quite convenient and efficient, provided that the gel has a sufficiently open structure to be able to absorb proteins efficiently ***(20)***. Coupled with the intrinsic high capacity of IPG gels, this procedure enables to easily separate milligram amounts of protein ***(20,37)***.

3.1.2. Solubility at the Isoelectric Point

This is usually the second critical point for IEF. The isoelectric point is the pH of minimal solubility, mainly because the protein molecules have no net electrical charge. This abolishes the electrostatic repulsion between protein molecules, which maximizes in turn protein aggregation and precipitation.

The horizontal comet shapes frequently encountered for major proteins and for sparingly soluble proteins often arise from such a near-isoelectric precipitation. Such isoelectric precipitates are usually easily dissolved by the SDS solution used for the transfer of the IEF gel onto the SDS gel, so that the problem is limited to a loss of resolution, which however precludes the separation of high amounts of proteins.

The problem is however more severe for hydrophobic proteins when an IPG is used. In this case, a strong adsorption of the isoelectric protein to the IPG matrix seems to occur, which is not reversed by incubation of the IPG gel in the SDS solution. The result is severe quantitative losses, which seem to increase with the hydrophobicity of the protein and the amount loaded ***(38)***. The sole solution to this serious problem is to increase the chaotropicity of the medium used for IEF, by using both urea and thiourea as chaotropes ***(21)***.

The benefits of using thiourea-urea mixtures to increase protein solubility can be transposed to conventional, carrier ampholyte-based focusing in tube gels with minor adaptations. Thiourea strongly inhibits acrylamide polymerization with the standard temed/persulfate system. However, photopolymerization with methylene blue, sodium toluene sulfinate and diphenyl iodonium chloride ***(39)*** enables acrylamide polymerization in the presence of 2M thiourea without any deleterious effect in the subsequent 2D ***(40)*** so that higher amounts of proteins can be loaded without loss of resolution ***(40)***.

3.1.3 The Epitome in Solubilization Problems: Membrane Proteins

Biological membranes represent a prototype of difficult samples for 2-D electrophoresis. They contain high amounts of lipids, which are troublesome compounds for 2-D electrophoresis. In addition, many membrane proteins are highly hydrophobic, and therefore very difficult to keep in aqueous solution, even with the help of chaotropes and detergents. Thus, membrane proteins have often been reluctant to 2-D electrophoretic analysis ***(41)***. In fact, it has been shown that typical membrane proteins with multiple transmembrane helices are resistant to solubilization by chaotrope-detergent mixtures, even using thiourea ***(42)***. For such proteins, the development of new detergents proved necessary ***(42,43)***. Even if some successes have been obtained ***(42–45)***, it is still obvious that many membrane proteins are not properly solubilized and focused with the chemicals described up to now ***(46)***. Therefore, further development is still needed to improve the representation of membrane proteins in 2-D maps. However, the problem of isoelectric precipiation may prevent definitively proper solubilization of membrane proteins in IEF-based 2-D electrophoretic systems

4. Concluding Remarks

Although this outline chapter has mainly dealt with the general aspects of solubilization, the main concluding remark is that there is no universal solubilization protocol. Standard urea-reducer-detergent mixtures usually achieve disruption of disulfide bonds and noncovalent interactions. Consequently, the key issues for a correct solubilization is the removal of interfering compounds, blocking of protease action, and disruption of infrequent interactions (e.g., severe ionic bonds). These problems will strongly depend on the type of sample used, the proteins of interest and the amount to be separated, so that the optimal solubilization protocol can vary greatly from a sample to another.

However, the most frequent bottleneck for the efficient 2-D separation of as many and as much proteins as possible does not lie only in the initial solubilization but also in keeping the solubility along the IEF step. In both fields, the key feature is the disruption of hydrophobic interactions, which are responsible for most, if not all, of the precipitation phenomena encountered during IEF. This means improving solubility during denaturing IEF will focus on the quest of ever more powerful chaotropes and detergents. In this respect, the use of thiourea has proven to be one of the keys to increase the solubility of proteins in 2-D electrophoresis. It would be nice to have other, even more powerful chaotropes. However, testing of the chatropes previously described as powerful ***(22)*** has not led yet to improvement in protein solubilization (T. Rabilloud, unpublished results). One of the other keys for improving protein solubilization is the use of as powerful detergent or detergent mixtures as possible. Among a complex sample, some proteins may be well denatured and solubilized by a given detergent or chaotrope, while other proteins will require another detergent or chaotrope (e.g., in ***[42]***). Consequently, the future of solubilization may still be to find mixtures of detergents and chaotropes able to cope with the diversity of proteins encountered in the complex samples separated by 2-D electrophoresis.

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12

Mouse and Human Tissue Sample Preparation for 2-D Electrophoresis

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1. Introduction

The protocol for extracting proteins from mouse and human tissues (organs) described in this chapter adheres to a strategy that is based on a rationale to include all protein species of a particular tissue in a set of samples which are suitable for two dimensional electrophoresis (2-DE), particularly for large gel 2-DE described in Chapter 32 ***(1)***. The extraction procedure is one of the most important steps to maintain reproducibility in 2-D gel based proteomics ***(2)***. The ultimate goal is resolution and visualization of all these protein species in 2-DE gels. This aim determines some features of our tissue extraction procedure for retrieving proteins. Since the last publication of our comprehensive protein extraction protocol ***(3)*** we found that an extraction protocol for total cell or tissue proteins may be sufficient for many more users than a protocol for fractionated extraction ***(4)***. Still, fractionated extraction has also a number of applications, especially when only certain classes of proteins such as cytoplasmic, membrane or nuclear proteins are of interest.

1.1. Total Extraction of all Cellular Proteins

Extraction of total proteins at once insures that no distribution artefacts occur as may be seen when proteins are fractionated into different subcellular fractions. Therefore the total amount of a given protein is determined within each protein sample investigated. In addition, the total extraction procedure is much simpler, takes less time and is highly compatible with new protein visualization techniques such as differential in gel electrophoresis (DIGE). This extraction procedure can be applied to almost any tissue, cell culture or subcellular components. No ultracentrifugation steps are involved which avoids protein precipitation and

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elimination during sample preparation. The total extraction procedure is based on adding protease inhibitors, detergent (CHAPS), urea/thiourea in very close succession to solubilze proteins in a tissue/cell as much as possible and keep them from degradation. Grinding the deep frozen tissue and sonication are the second instrumental part in the solubilization process. DNA-bound proteins are recovered by DNAse treatment. In this way all proteins are solubilized in one fraction without precipitation and fractionation steps.

1.2. Fractionated Extraction of Cellular Proteins

On the other hand, using the fractionation procedure the many different protein species of a tissue can be distributed over several 2-DE gels and this certainly increases resolution. However, a postulate is that fractionation of tissue proteins results in fraction-specific proteins. Usually, cell fractionation is performed with the aim of isolating special cell organelles (nuclei, mitochondria) or cell structures (membranes). Proteins are then extracted from these subcellular fractions. This procedure, however, includes washing steps to purify cell fractions and eliminate cell components which are not of interest and cell residues which are rejected. Using fractionation this way, an *uncontrolled* loss of proteins is unavoidable.

The tissue fractionation procedure described in this chapter renounces the isolation of defined cell components using washing and precipitation steps. This allows us to avoid any selective loss of proteins which is of utmost importance for all protein extraction protocols. Mouse (human) tissues (liver, brain, heart) are fractionated into three fractions: (1) The "supernatant I+II" (SI+II) containing the proteins soluble in buffer (cytoplasmic and nucleoplasmic proteins), (2) the "pellet extract" (PE) containing the proteins soluble in the presence of urea and CHAPS (proteins from membranes and other structures of the cells and cell organelles), and (3) the "pellet suspension" (PS) containing proteins released by DNA digestion (histones and other chromosomal proteins). The SI+II fraction is obtained by homogenization, sonication and centrifugation of tissue and reextraction of pellet (I) and the combination of the two supernatants gained in this way. The solution thus obtained is the first protein sample. The pellet (II) that remained is extracted with urea and CHAPS and the homogenate is centrifuged. The supernatant is the PE fraction and constitutes the second protein sample. The final pellet (III) is suspended into buffer containing benzonase, a DNA digesting enzyme. The pellet suspension (PS fraction) is the third protein sample. It is applied to 2-DE without further centrifugation.

Care is taken during the whole procedure to avoid any loss of material. In spite of this caution, some material may be lost, for example by transfer of the pulverized, frozen tissue from the mortar to a tube or by removing the glass beads from the sonicated homogenate. However, this does not lead to a preferential loss of certain protein species or protein classes.

1.3. Important Considerations When Using Protein Extraction Procedures

Generally, it is evident that the best conditions for keeping proteins stable and soluble are present in living cells ***(1,2)***. Therefore, an important principle of our tissue extraction procedure is to extract proteins, using conditions as close to the natural environment in the cell as possible. That means keeping ionic strength of the tissue homogenate at 150-200 m*M*, the pH in a range of 7.0-7.5, protein concentration high, and protecting the proteins against water by adding glycerol to the buffer. Generally, the best conditions for the first tissue extraction step would be if an addition of diluent that disturbs the natural protein concentrations and milieu of the cell were avoided. We prepared a pure cell sap from a tissue by homogenizing it without additives (except for protease inhibitor solutions added in small volumes) followed by high speed centrifugation and extracted the pellet that resulted successively in increasing amounts (0.5, 1 or 2 parts) of buffer. The series of protein samples obtained were separated by 2-DE and the patterns compared. The results showed that by increasing the dilution of proteins the number of spots and their intensities decreased in the lower part of the 2-DE patterns and increased in the upper part. The same phenomenon, but less pronounced, was observed even when the cell sap was diluted successively. Apparently, low molecular weight proteins are best dissolved in the pure cell sap and, presumably, tend to precipitate in more diluted extracts. In contrast, high molecular weight proteins dissolve better in more diluted samples. This effect was most pronounced in protein patterns from liver and not obvious in patterns from heart muscle. This is probably due to the high protein concentration in the liver cell sap that is not reached in extracts of other organs.

The dependence of protein solubility on the molecular weight of proteins is obscured in 2-DE patterns by another effect that causes a similar phenomenon. An increasing protein concentration of the first tissue extract leads to a higher activity of proteases released by breaking cellular structure by homogenization. Protein patterns from pure liver cell sap, extracted without protease inhibitors (but even with inhibitors) showed an enormous number of spots in the lower part of the gel and a rather depleted pattern in the upper part. In the pH range around pH 6 protein spots disappeared almost completely, in the upper as well as in the lower part, suggesting that these proteins are most sensitive to degradation by proteases. By extracting tissue or the first pellet with increasing amounts of buffer, the 2-DE pattern (spot number and intensity) shifted from the lower part to the upper part of the gel. Again, this observation was made particularly in liver.

The consequence of these observations for our total as well as our fractionated protein extraction procedure was to maintain the total extract or to obtain the supernatant I and II at concentrations that keep all the soluble proteins in solution but do not reach a level where proteases cannot be inhibited effectively anymore. As a consequence, we introduced buffer factors which adjust the concentration of the different extracts of each organ. The optimum protein concentrations in the extracts and therefore the buffer factors were determined experimentally. An optimum was reached when a maximum number of spots was present in the upper as well as in the lower part of a 2-DE protein pattern. In addition, the region close to pH 6 should not be depleted of spots, a process starting from the top.The methods described in the following sections were developed with mouse tissues but were found to be applicable to corresponding human tissues, cell culture cells and subcellular fractions as well.

2. Materials

2.1. Equipment

1. A small apparatus is preferred for performing sonication in a water-bath (Transsonic 310, FAUST, Singen, Germany).
2. Glass beads added to tissue samples for sonication: Size (diameter) of glass beads should be 2.0- 2.5 mm. A factor 0.034 was calculated for this bead size (*see* **Note 1**).
3. Mortar and pestle: Build and size of this equipment are shown in **Fig. 1**. Mortar and pestle are manufactured of agate or glass (WITA GmbH, Teltow, Germany). Glass

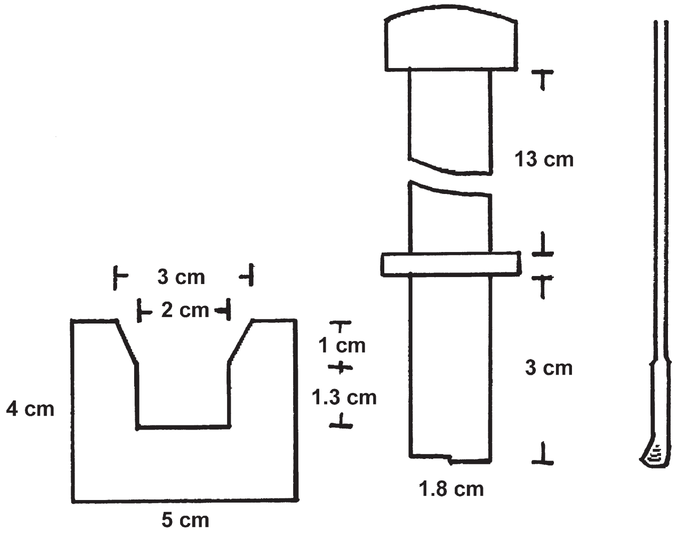

Fig. 1. Special equipment for pulverization of frozen tissue. **(A)** Glass mortar and plastic pestle. **(B)** Spatula used to transfer frozen tissue from a mortar to the test tube. A regular spatula was moulded into a small shovel.

was found to be more stable in liquid nitrogen. Alternatively an electronic mortar grinder can be used (Mortar Grinder RM 200; RETSCH, Haan, Germany).

4. A small spatula is formed into a shovel by wrought-iron work (**Fig. 1**) and used to transfer tissue powder from a mortar to tubes.

2.2. Reagents

1. Buffer A: 20% glycerol, 100 m*M* KCl, 50 m*M* Tris-HCl, pH 7.1, filtered and aliquoted into 1,000 µL portions, and stored at −70°C.
2. Phosphate buffer, pH 7.1: Add 67 mL 200 m*M* Na_2HPO_4 to 33 mL 200 m*M* NaH_2PO_4.
3. Buffer B: 20 % glycerol, 200 m*M* KCl, 100 m*M* phosphate buffer, pH 7.1, filtered, aliquoted *precisely* into 900 µL portions and stored at −70°C. When used, 100 µL of an aqueous CHAPS solution is added. The CHAPS concentration in this aqueous solution is calculated (*see* **Table 1**) so that the pellet II/buffer homogenate (*see* **Table 1**) contains 4.5% CHAPS. This concentration was found to be best when 2-DE patterns are compared. The concentration was determined empirically using protein samples containing different amounts of CHAPS.
4. Buffer C: 1 m*M* $MgSO_4$, 50 m*M* Tris-HCl, pH 8.0 (I mg of pellet III homogenised in 1 mL of this buffer gives a concentration of 1 mM $MgSO_4$. The final solution is filtered, aliquoted into 1 ml portions, and stored at −70°C.
5. Buffer P: 7.7 % glycerol (final concentration in sample if buffer factor is 1.6), 50 m*M* KCl, 50 m*M* Tris-HCl, pH 7.5. The final solution is filtered and aliquoted into 900 µL units and stored at −70°C.
6. Buffer P-$MgCl_2$: 5 m*M* $MgCl_2$ in buffer P. Final solution is aliquoted into 20 µL units and stored at −70°C.
7. Protease inhibitor 1A: One tablet of Complete™ (ROCHE, Mannheim, Germany) is dissolved in 2 ml buffer A (according to the manufacturer's instructions) and the resulting solution aliquoted into 50, 80 and 100 µL units. Inhibitor 1B was prepared in the same way but using buffer B (900 µL buffer + 100 µL H_20) and aliquoted into 30 and 50 µL units. Inhibitor 1C was prepared in the same way as Inhibitor 1A but using buffer P. Inhibitor 2 (pepstatin A): Prepared as a stock solution (9.603mg/100mL ethanol) and aliquoted into 100 µL portions. All inhibitor solutions are stored at −70°C.
8. DTT-solution: 2.16 g DTT is dissolved in 10 mL bidistilled water. The solution is aliquoted into 100 µL portions and stored at −70°C.
9. Sample diluent: Buffer P-$MgCl_2$. The solution is aliquoted into 250 µL units and stored at −70°C.

3. Methods

3.1. Extraction of Total Proteins

3.1.1. Dissection of Mouse Tissue

To obtain good results with protein extraction harvest organs rapidly and remove all contaminating material such as hair and blood.

Table 1
Total Tissue Protein Extraction Protocol

Liver	Brain	Heart
Homogenization		
– Liver tissue (25–50 mg)	– Brain tissue (25–150 mg)	– Heart tissue (25–150 mg)
– Buffer P-CHAPS	– Buffer P-CHAPS	– Buffer P-CHAPS
56 mg CHAPS	65 mg CHAPS	65 mg CHAPS
47 mg bidestilled water	38 mg bidestilled water	38 mg bidestilled water
+ 900 µL buffer P		
- P-CHAPS (2.5 × mg liver tissue)	- P-CHAPS (1.6 × mg brain tissue)	- P-CHAPS (1.6 mg heart tissue)
Σ_1 (mg liver tissue + mg P-CHAPS)	Σ_1 (mg brain tissue + mg P-CHAPS)	Σ_1 (mg heart tissue + mg P-CHAPS)
Inhibitor 1C (Σ_1 × 0.08)	Inhibitor 1C (Σ_1 × 0.08)	Inhibitor 1C (Σ_1 × 0.08)
Inhibitor 2 ((Σ_1 × 0.01)	Inhibitor 2 ((Σ_1 × 0.01)	Inhibitor 2 ((Σ_1 × 0.01)
Σ_2 (mg Σ_1 + mg Inhibitor 1C + mg Inhibitor 2)	Σ_2 (mg Σ_1 + mg Inhibitor 1C + mg Inhibitor 2)	Σ_2 (mg Σ_1 + mg Inhibitor 1C + mg Inhibitor 2)
Sonication		
Number of glass beads (Σ_2 × 0.034)	Number of glass beads (Σ_2 × 0.034)	Number of glass beads (Σ_2 × 0.034)
Sonication, 6 × 10 sec	Sonication, 6 × 10 sec	Sonication, 12 × 10 sec
Σ_3 (weight of sample determined after sonication)	Σ_3 (weight of sample determined after sonication)	Σ_3 (weight of sample determined after sonication)
Stirring		
4°C; 15 min	4°C; 15 min	4°C; 30 min
Benzonase treatment		
P-$MgCl_2$ (Σ_3 × 0.021)	P-$MgCl_2$ (Σ_3 × 0.021)	P-$MgCl_2$ (Σ_3 × 0.021)
Benzonase (Σ_3 × 0.025) 4 °C; 15 min	Benzonase (Σ_3 × 0.025) 4 °C; 15 min	Benzonase (Σ_3 × 0.025) 4 °C; 15 min
Σ_4 (weight of sample determined after benzonase treatment)	Σ_4 (weight of sample determined after benzonase treatment)	Σ_4 (weight of sample determined after benzonase treatment)
Control factor		
Liver tissue/Σ_4	Brain tissue/Σ_4	Heart tissue/Σ_4

Protein concentration		
Aliquot 5 µL	Aliquot 5 µL	Aliquot 5 µL
Σ_5 (weight of sample determined after aliqout)	Σ_5 (weight of sample determined after aliqout)	Σ_5 (weight of sample determined after aliqout)
Urea/thiourea		
Urea 6M ($\Sigma_5 \times 0.78$)	Urea 6M ($\Sigma_5 \times 0.78$)	Urea 6M ($\Sigma_5 \times 0.78$)
Thiourea 2M ($\Sigma_5 \times 0.3$)	Thiourea 2M ($\Sigma_5 \times 0.3$)	Thiourea 2M ($\Sigma_5 \times 0.3$)
Stirring RT, 30 min	Stirring RT, 30 min	Stirring RT, 30 min
Aliquot DIGE (min. 20 µL)	Aliquot DIGE (min. 20 µL)	Aliquot DIGE (min. 20 µL)
Σ_6 (weight of sample determined after aliquot DIGE)	Σ_6 (weight of sample determined after aliquot DIGE)	Σ_6 (weight of sample determined after aliquot DIGE)
DTT ($\Sigma_6 \times 0.1$) RT, 5 min	DTT ($\Sigma_6 \times 0.1$) RT, 5 min	DTT ($\Sigma_6 \times 0.1$) RT, 5 min
Ampholin ($\Sigma_6 \times 0.1$)	Ampholin ($\Sigma_6 \times 0.1$)	Ampholin ($\Sigma_6 \times 0.1$)
Σ_7 (weight of sample after DTT + Ampholin addition)	Σ_7 (weight of sample after DTT + Ampholin addition)	Σ_7 (weight of sample after DTT + Ampholin addition)
IEF (1-D)		
5 µL/gel	5 µL/gel	5 µL/gel

3.1.1.1. DISSECTION OF MOUSE LIVER

1. Kill the mouse by decapitation. Thereby, the body is allowed to bleed. The following steps are performed in a cold room:
2. Cut open the abdomen and through the *vena femoralis* on both sides and perfuse the liver with 5 ml saline (0.9% NaCl solution).
3. Dissect the complete liver from the body, remove the gall bladder without injuring it and separate the liver into its different lobes. The central part of each lobe, i.e. the region where blood vessels enter the liver lobe, is cut off as are the remainders of other tissues (diaphragm, fascia).
4. Cut the liver lobes into 2-4 pieces, rinse in ice-cold saline and briefly immerse it there. Immediately after this step the next organ (e.g. brain) is prepared from the same animal, if desirable, and processed to the same stage of preparation as liver.
5. Cut the liver pieces into smaller pieces (about 5 × 5 mm) and transfer each piece to filter paper, immerse into liquid nitrogen, and put into a screw-cap tube where all pieces are collected. During preparation time, tubes are kept in a liquid nitrogen containing box and afterwards stored at –70°C.

3.1.1.2. DISSECTION OF MOUSE BRAIN

1. Kill mouse by decapitation. The following steps are performed in a cold room. If several organs have to be taken from the same animal, start with the brain.
2. Cut off the skin of the head and open the cranium starting from the spinal canal proceeding in a frontal direction. Break the cranial bones apart to expose the brain. Remove the brain including both *bulbi olfactorii* and a small part of the spinal cord. Transfer the brain into a petri dish containing ice-cold saline. Remove any blood vessels and blood from the outside of the brain.
3. As desired, cut the brain to retrieve the desired brain regions or in half along the *corpus callosum*, place the pieces on filter paper and then individually immerse into liquid nitrogen and collect in a screw-capped tube. Keep the tubes in liquid nitrogen during tissue harvest and finally store at –70°C.

3.1.1.3. DISSECTION OF MOUSE HEART

1. Kill mouse by decapitation. The following steps are performed in a cold room.
2. Open the thorax and remove the heart. Place the heart into a petri dish containing ice-cold saline. Cut off both atria and open the *ventriculi* to remove any blood and blood clots.
3. Dry the heart on filter paper, freeze in liquid nitrogen and store in a screw-capped tube at –70°C.

3.1.2. Total Extraction of Liver Proteins

1. Fill frozen liver pieces into a small plastic tube of known weight and weigh quickly without thawing. The amount of liver tissues used for extraction should be between 50 and 150 mg.

2. Place a mortar, pestle and a small metal spoon into a styrofoam box that contains liquid nitrogen to a level not exceeding the height of the mortar. The mortar should be pre-cooled for at least 3–4 min. Pre-cool pestle and spatula.
3. Put frozen liver pieces into the mortar, and add buffer P, inhibitor 1C and 2. The required volumes of each of the solutions are calculated as indicated in **Table 1**. The precise amount of each solution is pipetted as a droplet onto a small spoon-like spatula that was kept in the N_2-box before use. The solution is immediately frozen into an ice bead that can easily be transferred into the mortar.
4. Slowly grind all frozen components in the mortar to powder. Care should be taken that small pieces of material do not jump out of the mortar when starting to break up the solid frozen material with a pestle.
5. Transfer the powder into a pre-frozen 2 ml Eppendorf tube using a special spatula (**Fig. 1**). Forceps are used to freeze the tube briefly in N_2 and then to hold the tube close to the mortar in the N_2-box. Care is taken that no powder is left in the mortar or on the pestle. For collection of this powder always use the same type of plastic tube. This contributes to reproducibility of the subsequent sonication step. Compress the powder collected in the tube by gently knocking the tube against the mortar. The powder can be stored at −70°C or immediately subjected to sonication.
6. For sonication a predetermined number of glass beads (*see* **Table 1** and **Note 1**) is added to the sample and the powder is then thawed and kept immersed in ice. Sonication is performed in an ice-cold water-bath. The fill-height of the water is critical for the sonication effect and should always be at the level indicated in the instruction manual of the manufacturer. Furthermore, when dipping the sample tube into the water, it is important to do this at the "sonication center" visible by a concentric water surface motion which appear when holding the tube into the water. We prefer a small sonication apparatus that forms only one sonication center (*see* **Subheading 2.1**). Sonication is performed for 10 sec. Immediately afterwards the sample is stirred with a thin wire for 50 sec with the tube still remaining in the ice water. The tube is then kept immersed in ice for 1 min. Now the next sonication circle is started, until a total of 6 two-minute steps (*see* **Note 1**). After sonication, the tubes are turned upside down and punctured with a steel needle to generate a 2 mm diameter opening. The holes should not be larger than that to keep the glass beads from crossing over. A second tube is attached to the bottom of the first. Both tubes are transferred into a centrifuge using other samples or dummy tubes for balancing. Tubes are quickly centrifuged at 2,000 *g* for 1 min. The sample is thereby transferred in to the lower tube whereas the glass beads remain in the upper.
7. Add a small bar magnet and slowly stir homogenate at 4°C for 15 min.
8. Add benzonase (Merck, Darmstadt, Germany) and P-$MgCl_2$. Slowly stir the homogenate for 15 min at 4°C (DNA digestion).
9. Determine control factor (**Table 1** and **Note 2**).
10. Remove aliquot for determination of protein concentration. (***Note***: When sample volume is very low, remove aliquot only after addition of urea/thiourea.)
11. Add urea and thiourea to the homogenate. The amounts indicated in **Table 1** are transferred into a capped 1.5 mL tube. The tube is sealed and turned up side down. Now the bottom of the tube is cut away by scissors and the tube is put on top of

the 2 mL tube containing the homogenate. A quick-spin transfers the urea/thiourea completely into the homogenate (1 min, 1,000g). The homogenate is stirred for about 30 min until the urea/thiourea is dissolved. Now the aliquot for DIGE can be sampled (**Table 1** and **Note 3**).

12. Add DTT and stir homogenate for at least 5 min then add Ampholin 2-4. Now, carefully remove the small bar magnet. Now aliquot samples to omit freeze and rethaw cycles. Usually samples need to be diluted (most commonly 1:2) by sample dilution solution (Buffer P-$MgCl_2$) before application to isoelectric focusing (IEF).
13. Samples are quick-frozen and stored at −70°C.

3.1.3. Extraction of Total Proteins from Other Tissues

In general, the total extraction protocol differs only in buffer factors and the repeats of sonication (**Table 1**). Only very special tissue such as lens would show deviations. In our experience lens does not require DNAse treatment.

3.2. Fractionated Extraction of Proteins

3.2.1. Fractionated Extraction of Liver Proteins

3.2.1.1. EXTRACTION OF LIVER PROTEINS SOLUBLE IN BUFFER (SUPERNATANT I + II)

1. Fill frozen liver pieces into a small plastic tube of known weight and weigh quickly without thawing. The weight of the liver tissues should be between 25 and 260 mg (for lower amounts *see* **Note 4**).
2. Place a mortar, pestle and a small metal spoon into a styrofoam box that contains liquid nitrogen to a level not exceeding the height of the mortar.
3. Put frozen liver pieces into the mortar, and add buffer A, inhibitor 1A and 2. The required volumes of each of the solutions are calculated as indicated in **Table 2.** The precise amount of each solution is pipetted as a droplet onto the spoon that was kept in the N_2-box before use. The solution immediately is immediately frozen into an ice bead that can easily be transferred into the mortar.
4. Grind all frozen components in the mortar to powder. Care should be taken that small pieces of material do not jump out of the mortar when starting to break up the hard frozen material with the pestle.
5. Transfer the powder into a 2 mL Eppendorf tube using a special spatula (**Fig. 1**). Forceps are used to freeze the tube briefly in N_2 and then to hold the tube close to the mortar in the N_2-box. Care is taken that no powder is left in the mortar or on the pestle. For collection of this powder always use the same type of plastic tube. This contributes to reproducibility of the subsequent sonication step. Compress the powder collected in the tube by gently knocking the tube against the mortar. The powder can be stored at −70°C or immediately subjected to sonication.
6. For sonication a predetermined number of glass beads (*see* **Table 2** and **Note 1**) is added to the sample and the powder is then thawed and kept immersed in ice. Sonication is performed in an ice-cold water-bath. The fill-height of the water is critical

Table 2
Fractionated Tissue Protein Extraction Protocol

SI+II fraction: Liver		Brain	Heart
– Liver pieces	250 mg[2] (A)[3]	– 25-250 mg fine pieces	– 100-130 mg (total heart)
Buffer A (liver mg × 1.5)[1]	375 µL	No buffer	Buffer A (liver mg × 1.0)
Σ_1	625		
Inhibitor 1A ($\Sigma_1 \times 0.08$)	50 µL	Inhibitor 1A ($\Sigma_1 \times 0.08$)	Inhibitor 1A ($\Sigma_1 \times 0.08$)
Inhibitor 2 ($\Sigma_1 \times 0.01$)	12.5 µL	Inhibitor 2 ($\Sigma_1 \times 0.01$)	Inhibitor 2 ($\Sigma_1 \times 0.01$)
Σ_2	688		
– Liver powder		– Brain powder	– Heart powder
– Sonication, 6 × 10 sec		– No sonication	– Sonication, 12 × 10 sec
Number of glass beads ($\Sigma_2 \times 0.034$)	23		
– Centrifugation		– Centrifugation	– Centrifugation
Supernatant I store frozen		Supernatant I store frozen	Supernatant I store frozen
– Pellet I weigh	110 mg[4]	– Pellet I weigh	– Pellet I weigh
Buffer A (pellet I mg × 2)	220 µL	Buffer A (pellet I mg × 0.5)	Buffer A (pellet I mg × 1.0)
Σ_3	330		
Inhibitor 1A ($\Sigma_3 \times 0.08$)	26.4 µL	Inhibitor 1A ($\Sigma_3 \times 0.08$)	Inhibitor 1A ($\Sigma_3 \times 0.08$)
Inhibitor 2 ($\Sigma_3 \times 0.01$)	6.6 µL	Inhibitor 2 ($\Sigma_3 \times 0.01$)	Inhibitor 2 ($\Sigma_3 \times 0.01$)
– Pellet powder		– Pellet powder	– Pellet powder
		– Sonication, 6 × 10 sec	
		Number of glass beads $[\Sigma_3 + (\Sigma_3 \times 0.08) + (\Sigma_3 \times 0.01)] \times 0.034$	
– Stirring		– No stirring	– Stirring
– Centrifugation		– Centrifugation	– Centrifugation
Supernatant II add to I		Supernatant II add to I	Supernatant II add to I
Supernatant I+II weigh	628 mg [5] (B)[3]	Supernatant I+II weigh	Supernatant I+II weigh
– Aliquot of supernatant I+II (store rest of supernatant I+II frozen)	50 µL	– Aliquot of supernatant I+II (store rest of supernatant I+II frozen)	– Aliquot of supernatant I+II (store rest of supernatant I+II frozen)
Urea (50 µL × 1.08)	54 mg	Urea (50 µL × 1.08)	Urea (50 µL × 1.08)

(continued)

Table 2 (continued)

SI+II fraction: Liver		Brain	Heart
DTT solution (50 µL × 0.1)	5 µL	DTT solution (50 µL × 0.1)	DTT solution (50 µL × 0.1)
Ampholyte pH 2-4 (50 µL × 0.1)	5 µL	Ampholyte pH 2-4 (50 µL × 0.1)	Ampholyte pH 2-4 (50 µL × 0.1)
Final volume of 50 µL supernatant plus additives	100 µL	Final volume of 50 µL supernatant plus additives	Final volume of 50 µL supernatant plus additives
Diluent	100 µL	No diluent	Diluent
Supernatant I+II, ready for use, store frozen in aliquotes	200 µL	Supernatant I+II, ready for use, store frozen in aliquotes	Supernatant I+II, ready for use, store frozen in aliquotes
– 2-D electrophoresis	8 µL/gel	9 µL/gel	6 µL/gel
PE fraction: Liver		Brain	Heart
– Pellet II weight	92 mg ^{4} (C)^3	– Pellet II weight	– Pellet II weight
Buffer B/CHAPS (pellet II mg × 1.6)	147 µL	Buffer B/CHAPS (pellet II mg × 1.4)	Buffer B/CHAPS (pellet II mg × 2.2)
900 µL buffer B		900 µL buffer B	900 µL buffer B
73 mg CHAPS (displace 69 µL)		77 mg CHAPS	65 mg CHAPS
31 µL bidistilled water		27 µL bidistilled water	38 µL bidistilled water
1000 µL buffer B/CHAPS		1000 µL buffer B/CHAPS	1000 µL buffer B/CHAPS
Σ_4	239		
Inhibitor 1B (Σ_4 × 0.08)	19 µL	Inhibitor 1B (Σ_4 × 0.08)	Inhibitor 1B (Σ_4 × 0.08)
Σ_5	19+147^6 = 166		
– Pellet powder		– Pellet powder	– Pellet powder
Stirring		Stirring	Stirring
– Urea [(pellet II mg × 0.3) + Σ_5] × 1.08	207 mg	– Urea [(pellet II mg × 0.56) + Σ_5] × 1.08	– Urea [(pellet II mg × 0.25) + Σ_5] × 1.08
DTT solution [(pellet II mg × 0.3) + Σ_5] × 0.1	19 µL	– DTT solution [(pellet II mg × 0.56) + Σ_5] × 0.1	– DTT solution [(pellet II mg × 0.25) + Σ_5] × 0.1
Stirring		Stirring	Stirring
– Centrifugation		– Centrifugation	– Centrifugation

Supernatant III weigh	375 mg[5] (D)[3]	Supernatant III weigh	Supernatant III weigh
Ampholyte pH 2 – 4 (Serva) (supernatant III mg × 0.0526)	20 µL	Ampholyte pH 2 – 4 (Serva) (supernatant III mg × 0.0526)	Ampholyte pH 2 – 4 (Serva) (supernatant III mg × 0.0526)
Pellet extract, ready for use, store frozen in aliquots		Pellet extract, ready for use, store frozen in aliquots	Pellet extract, ready for use, store frozen in aliquots
– 2-D electrophoresis	8 µL/gel	8 µL/gel	7 µL/gel
PS fraction: Liver		Brain	Heart
– Pellet III	69 mg[4]	– Pellet III	– Pellet III
Buffer C (pellet III mg × 1.0)	69 µL	Buffer C (pellet III mg × 1.0)	Buffer C (pellet III mg × 1.0)
Σ_6	138		
– Pellet powder		– Pellet powder	– Pellet powder
– Benzonase (Merck) ($\Sigma_6 \times 0.025$)	3.5 µL	– Benzonase (Merck) ($\Sigma_6 \times 0.025$)	– Benzonase (Merck) ($\Sigma_6 \times 0.025$)
Stirring		Stirring	Stirring
Σ_7	3.5+69[7] = 73		
– Urea ($\Sigma_7 \times 1.08$)	79 mg	– Urea ($\Sigma_7 \times 1.08$)	– Urea ($\Sigma_7 \times 1.08$)
DTT solution ($\Sigma_7 \times 0.1$)	7.3 µL	DTT solution ($\Sigma_7 \times 0.1$)	DTT solution ($\Sigma_7 \times 0.1$)
Stirring		Stirring	Stirring
Ampholyte pH 2-4 [(pellet III mg × 0.3) + Σ_7] × 0.1	9.4 µL	Ampholyte pH 2-4 [(pellet III mg × 0.56) + Σ_7] × 0.1	Ampholyte pH 2-4 [(pellet III mg × 0.25) + Σ_7] × 0.1
Pellet suspension, ready for use, store frozen in aliquots			
– 2-D electrophoresis	9 µL/gel	8 µL/gel	8 µL/gel

[1] All factors used in this table are explained in **Note 8**.

[2] This figure is given as an example. The amount of starting material may vary from 25 to 250 mg (*see* **Note 4**).

[3] B ÷ A = control value; D ÷ C = control value (*see* **Note 2**).

[4] This figure is provided as an example and is not the result of a calculation. The pellet weight varies because slight losses of material are unavoidable, even during very precise work.

[5] *See* **Footnote 4** for pellets; this also holds true for supernatants.

[6] Buffer B/CHAPS volume.

[7] Buffer C.

for the sonication effect and should always be at the level indicated in the instruction manual of the manufacturer. Furthermore, when dipping the sample tube into the water, it is important to do this at a "sonication center" visible by a concentric water surface motion which appear when holding the tube into the water. We prefer a small sonication apparatus that forms only one sonication center (*see* **Subheading 2.1**). The water must be kept ice-cold. Sonication is performed for 10 sec. Immediately afterwards the sample is stirred with a thin wire for 50 sec with the tube still remaining in the ice water. The tube is then kept immersed in ice for 1 min. Now the next sonication circle is started, until a total of 6 two-minute steps (*see* **Note 1**). After sonication, the tubes are turned upside down and punctured with a steel needle to generate a 2 mm diameter opening. The holes should not be larger than that to keep the glass beads from crossing. A second tube is attached to the bottom of the first. Both tubes are transferred into a centrifuge using other samples or dummy tubes for balancing. Tubes are quickly centrifuged at 2,000 *g* for 1 min. The sample is thereby transferred into the lower tube whereas the glass beads remain in the upper. The homogenate is then frozen in liquid nitrogen and can be stored at −70°C. Be careful to hold the tube at an angle of about 45° to decrease the difficulty of retrieving the frozen homogenate.

7. Detach the frozen homogenate in the tube from the wall by quickly knocking at the tube with a large screw driver. Transfer the frozen piece of sample into a preweighed centrifuge tube and briefly thaw the homogenate. Centrifuge for a maximum of 226,000 *g* for 30 min at 4°C.
8. Completely withdraw the supernatant (I) with a pasteur pipette and fill into a preweighed small test tube. The centrifuge tube is kept on ice and the pipette is put back into the tube again with the tip at the center of its bottom (***Note***: the pellet sticks to the wall if a fixed-angle rotor was used). In this position, remainders of the supernatant accumulate at the bottom of the tube and inside the pipette. Withdraw residual supernatant with the pipette and transfer it to the test tube. Now the supernatant is frozen in liquid nitrogen and stored at −70°C.
9. Weigh the pellet (I) left in the centrifuge tube on ice and add buffer A at an amount calculated as indicated in **Table 2**. Mix the pellet and buffer by vortexing, collect the homogenate at the bottom by a short spin, freeze in liquid nitrogen and store at −70°C, or process further immediately.
10. Transfer the homogenate from the centrifuge tube to the mortar after detaching the frozen homogenate from the wall by knocking onto the bottom of the tube. Grind the homogenate together with inhibitors 1A and 2 to powder as described above for the liver pieces. Transfer the powder back into the used centrifuge tube, taking care that no powder remains in the mortar.
11. Thaw the powder and slowly stir the homogenate for 45 min in a cold room.
12. Centrifuge the homogenate as described in **step 7** above.
13. Completely withdraw the supernatant (II) from the pellet. This is done in such a way that a white layer which partially covers the surface of the supernatant sinks unaffected onto the pellet. Collect the remainder of the supernatant as mentioned in **step 8.** Add supernatant II to supernatant I and thoroughly mix both solutions. Determine the weight of the total supernatant.

14. Take a 50 µL-aliquot from the total supernatant and mix with urea, DTT-solution and ampholyte pH 2-4, as indicated in **Table 2**. The final concentrations of these components are: 9 *M* urea, 70 m*M* DTT and 2% ampholytes. These three components should be added to the supernatant in the order indicated here and each component should be mixed and dissolved in the supernatant before adding the next. The final volume of this supernatant mixture is 100 µL. Add 100 µL sample diluent (*see* **Table 3**) and mix before IEF run.
15. The resulting solution is the final sample ("supernatant I + II", SI + II). Divide the sample into several portions, freeze each portion in liquid nitrogen and store at −70°C. Usually 8 µL of sample are applied to an IEF gel, if the large gel 2-D electrophoresis technique described in Chapter 11 is used (*see* **Note 5**). Freeze the remaining portion of the pure supernatant and store at at −70°C.
16. Determine the weight of the pellet (II). Collect the pellet at the bottom of the tube by a short spin, then freeze in liquid nitrogen and store at −70°C.

3.2.1.2. Extraction of the Pellet Proteins Soluble in the Presence of Urea and CHAPS (Pellet Extract)

1. Grind pellet II, buffer B/CHAPS and inhibitor 1B to powder in a mortar placed in liquid nitrogen (*see* **Subheading 3.2.1.1., steps 2-4**). The calculations for buffer and inhibitor volumes are provided in **Table 2**. Transfer the powder back to the centrifuge tube, avoid leaving any remainders in the mortar or on the pestle.
2. Thaw the powder mix and stir slowly for 60 min in a cold room (CHAPS solubilizes structural proteins).
3. Add urea (for the amount see **Table 2**) to the homogenate and stir the mixture for 45 min at room temperature (urea reaction). Some minutes after adding urea a large part of it is already dissolved. At this point add DTT solution (for the amount see **Table 2**).
4. Remove the magnet rod from the homogenate. At this step, also avoid any loss of homogenate. Centrifuge the homogenate at 17°C for 30 min at 50,000 rpm.
5. Completely withdraw the supernatant (III) with a Pasteur pipette and fill it into a small pre-weighed test tube. Collect the remainders of the supernatant as mentioned in **Subheading 3.2.1.1.**, step 8. Determine the weight of the supernatant.
6. Add ampholytes pH 2-4 (for amount *see* **Table 2**) to the supernatant and immediately mix solution.

Table 3
Sample Diluent

Component	Mixture	Final concentration	
Urea	1.08	g (=0.80 ml)	9.000 M
DTT-solution [a]	0.10	ml	0.070 M
Servalyt pH 2 – 4 (Serva)	0.10	ml	2.000 %
Bidistilled water	1.00	ml	50.000 %
Sample diluent	2.00	ml	

[a] *See* **Subheading 2.2,** item 5.

7. The resulting solution is the final sample (”pellet extract”, PE). Divide the sample into several portions, freeze each portion in liquid nitrogen and store at −70°C. Usually the volume of sample applied per IEF gel is 8 µL, if large gel 2-D electrophoresis (*see* Chapter 18) is used (*see* **Note 5**).
8. Determine the weight of the pellet (III). Collect the pellet on the bottom of the tube by a short spin, then freeze in liquid nitrogen and store at −70°C.

3.2.1.3. Suspension of the Remaining Pellet (Pellet Suspension)

1. Grind pellet III and buffer C to powder in a mortar as described in **Subheading 3.2.1.1.**, **steps 2-4.** The buffer volume is calculated as indicated in **Table 2**. The powder is transferred into a test tube, avoiding any loss of material.
2. Thaw powder and add benzonase (Merck, Darmstadt, Germany; for the amount *see* **Table 2**). Slowly stir the homogenate for 30 min in a cold room (DNA digestion).
3. Add urea (for amount *see* **Table 2**) and stir the homogenate at room temperature for another 30 min. During this time add DTT solution (for amount *see* **Table 2**) once the largest part of urea is dissolved. At the end of this period add ampholytes pH 2-4 (for amount *see* **Table 2**) and quickly mix with the homogenate.
4. The resulting solution is the final sample (”pellet suspension,” PS). It is frozen in liquid nitrogen and stored at −70°C. Usually 9 µL of sample is applied per IEF gel (if large gel 2-D electrophoresis is used; *see* Chapter 11 and **Note 6**). The sample still contains some fine undisolved material and is therefore transferred to the gel with a thin Pasteur pipette instead of a microliter syringe.

3.2.2. Fractionated Extraction of Brain Proteins

Frozen brain tissue is transferred into a mortar that was placed into a box containing liquid nitrogen, and crushed with the pestle to fine pieces. The crushed material is transferred completely back into test tubes so that one tube contains 25-250 mg of frozen tissue (weigh the tube without thawing the tissue).

This material is used to prepare the supernatant I + II, the pellet extract and the pellet suspension. The procedure follows that of liver extraction with some differences indicated in **Table 2**. One exception is that the tissue powder is produced without buffer and subjected to centrifugation without sonication so that a rather small amount of supernatant I results. Sonication is only performed with pellet I homogenate.

3.2.3. Fractionated Extraction of Heart Proteins

The supernatant I + II, the pellet extract and the pellet suspension are prepared from a single heart. The procedure is as described for liver with some modifications which are indicated in **Table 2**.

4. Notes

1. Sonication: Conditions for sonicating mouse tissue homogenates (liver, brain, heart) were optimized to break membranes of all cells and cell nuclei

of a tissue. Three parameters were varied in the experiments: the time of sonication, the number of sonication repeats and the number of glass beads added per volume of homogenate. The effect of the various parameters was determined by inspection of sonicated material under the microscope. Sonication for 10 sec increases the temperature of the homogenate from 0°C to 11-12°C. Therefore, sonication was not performed for more than 10 sec. Glass beads are essential for breaking cellular structures (membranes). Since most of the homogenates are rather viscuous fluids, the beads cannot flow freely and their addition is limited. For a given homogenate volume a certain number of glass beads is necessary to expose it evenly to the disrupting power of sonication. Due to viscosity, an increase in this number has not much effect. This number can be calculated. By standardization experiments we determined that a factor 0.034 is a good approximation for the optimal number of glass beads for a given volume of homogenate. It can be concluded from the above-mentioned that the only parameter which can be varied without harming the sample while still increasing the effect of sonication, was the number of repeats of the 10 sec sonication period. Under the conditions described in the Methods section the membranes of all cells were broken and no longer visible under the microscope. However, a certain number of intact nuclei were still detectable. Still, this shortcoming was not compensated for by increasing the number of sonication repeats because we assume that homogenization, stirring and high-speed centrifugation may eventually extract all nucleoplasm proteins. A more aggressive sonication procedure using a metal tip cannot be recommended. We observed heavily disturbed 2-DE patterns as a result of employing this technique: many protein spots disappeared depending on the extent of sonication and new spot series occurred in the upper part of the gel, apparently as a result of aggregation of protein fragments.

2. Control values: Control values were calculated for each sample prepared by the fractionated extraction procedure to monitor correctness and reproducibility of the preparation. The calculation of control values is shown in **Tables 1 and 2**. To visualize the calculation procedure an example from a real experiment is provided: From a series of 73 individual mouse hearts the SI+II fractions were prepared and the control values B÷A (*see* **Table 2**) were calculated: 60 samples yielded values between 1.97-2.10, three samples between 1.94-1.96, and six samples between 2.11-2.13. Four samples with largest deviations (1.90, 1.91, 2.16 and 2.24) were excluded from the investigation. The range 1.97-2.10 (mean 2.04 ± 0.04) was taken as reference value for preparation of mouse heart SI+II samples.
3. The extraction procedure was adapted for labeling with CyDyes. It is important that only urea and thiourea should be present in the sample for

labeling. DTT and ampholines are added only afterwards since both bind CyDyes. In addition, determination of the protein concentration is now mandatory since the amount of CyDyes for labeling is calculated based on the protein concentration. Therefore a 5 µL aliquot of the sample is specifically used for this purpose (**Table 1**, protein concentration). 50 µg protein labeled by 400 pMol CyDye works well for brain samples.

4. The tissue amount indicated (25 mg – 150 mg) yields enough sample to run a large number of 2-DE gels so that less rather than more material may be used. In cases in which only a very small amount of tissue is available (e.g. 2-5 mg heart biopsy samples, 10-12 mg of two mouse eye lenses, early mouse embryos) a total protein extract should be prepared instead of SI+II, PE and PS fractions. Small plastic tubes and a glass rod with a rough surface at the well fitting tip may serve as mortar and pestle.
5. Amount of protein applied per gel: The protein amount applied to IEF gels (*see* **Tables 1** and **2**), contains about 100 µg protein. There is, however, no need to determine the protein concentration of each sample prepared in order to get protein patterns of reproducible intensities. The concept of the procedure described here for extracting tissues was to keep the volume of the extracts in strong correlation to the amount of the starting material (tissue or pellet) which was extracted. Therefore, by working precisely, the final sample should always contain nearly the same protein concentration. This was confirmed by determining the protein concentrations of a large number of protein samples. Accordingly, the reproducibility of the pattern intensity depends on the precise sample volume applied to the gel – and, of course, on the protein staining procedure. The sample volumes per gel given in **Table 2** are adapted for silver staining protocols. As a general guideline one should take into account: decreasing the protein amount per gel and increasing the staining period is better than the vice versa; diluted samples using reasonable volumes are better than concentrated protein samples at a small volume since in the first case clogging is avoided.
6. Maximum resolution of tissue protein fractions by 2-D electrophoresis: **Fig. 2** shows 2-DE patterns of three protein fractions SI+II, PE and PS of mouse liver. The SI+II fraction reveals the highest number of protein spots.

Fig. 2. (continued) pattern and vice versa. Pellet-specific spots occur predominantly in the basic half, supernatant-specific spots more on the acid half of the pattern. The pellet suspension pattern reveals very basic proteins of a tissue extracts. Since IEF gels do not cover the entire basic pH range, these proteins cannot reach their isoelectric points. To prevent these proteins from accumulating at the basic end of a gel, the IEF run was shortened by 2 h at the 1000 V level. Consequently, the very basic proteins form streaks instead of focused spots. For evaluation of patterns in terms of spot number *see* **Notes 6** and **7**.

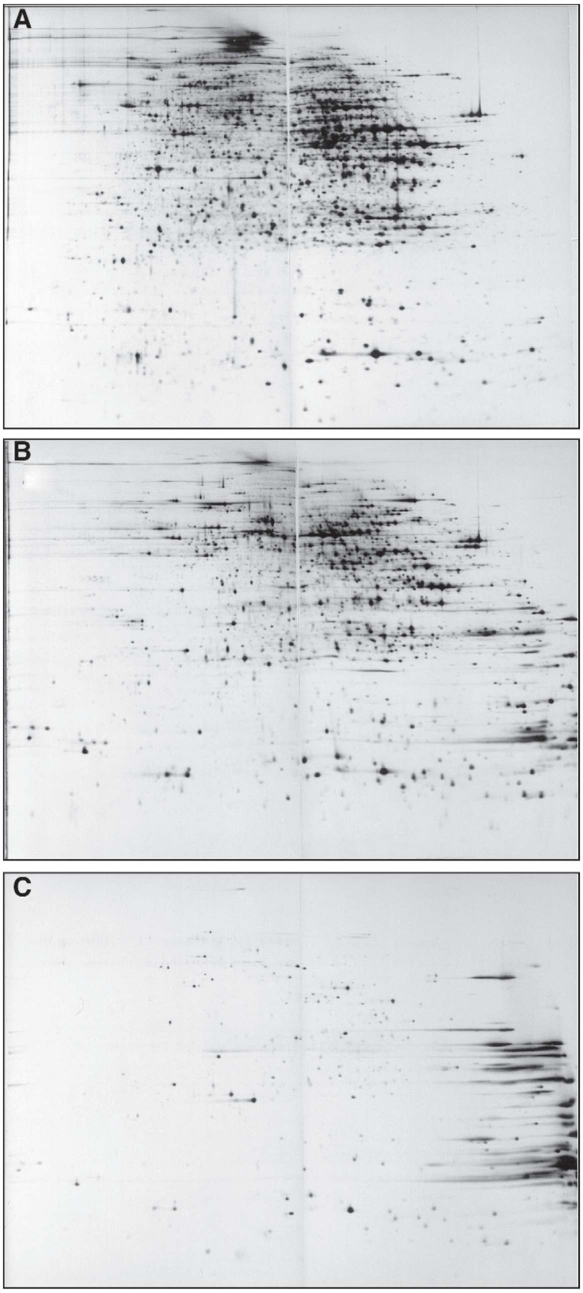

Fig. 2. 2-DE protein patterns from mouse liver. Tissue was fractionated into supernatant I + II (a), pellet extract (b), and pellet suspension (c) as described in **Subheading 3.1**. Three fractions were subjected to large gel 2-D electrophoresis (see Chapter 11). In the pellet extract pattern many protein spots were visible which are not present in the supernatant

When spots were counted visually, i.e. by placing a 2-DE gel on a light box and dotting each spot with a pencil **(3),** about 9200 proteins were detected in this fraction. The SI+II pattern of the brain revealed about 7700 proteins, that of the heart about 4800 protein spots. The high spot numbers reflect the high resolution of the large gel 2-DE (see Chapter 11), which reveals many weak spots between major spots. All these spots were counted with high precision and great care.

7. Effect of fractionated extraction of tissue proteins: The purpose of fractionating the proteins of a tissue was to increase the number of proteins detectable by 2-DE. This goal, however, would only be achievable if each fraction contained a notable number of proteins which are strongly fraction-specific, so that the total tissue proteins can be distributed over several gels. Comparison of the 2-DE patterns from the SI+II and PE fractions of the liver **(Fig. 2)** showed that the PE pattern revealed about 2000 protein spots not detectable among the 9200 spots of the SI+II pattern. The PS pattern revealed only about 70 additional spots. The PS protein spots represent classes of the most basic proteins (**Fig. 2**) and belong mainly to the chromosomal proteins (e.g. histones). Therefore, the PS fraction is only of interest when a class of very basic proteins is subject of the investigation. Considering the 2-DE patterns of the SI+II and PE fraction in more detail (**Fig. 3**), quite a number of very prominent spots can be observed which are visible in one pattern but do not occur, not even in trace amounts, in the others. At the same time, other spots revealing only low intensities are present in both patterns. This suggests that the phenomenon where many protein spots of the SN I+II pattern also occur in the PE pattern is not due to impurities of the pellet fraction by supernatant proteins caused by technical problems. We found, using centrifugation at different speeds and samples prepared with different degrees of viscosity, that an overlap in 2-DE patterns is due to the fact that most proteins exist solitarily as well as in protein-complexes. Apparently, protein complexes sink to the pellet at high speed and are later resolved again by detergent and urea extraction in the PE fraction. Taking into account all three fractions, the total liver proteins could be resolved into about 11,270 different proteins (polypeptide spots). This, however, does not mean that the protein sample preparation procedure described here – in combination with the 2-DE technique in Chapter 32 – revealed all proteins of the liver. Many proteins may exist in a tissue in undetectable amounts, and special fractionation procedures with subsequent protein concentration steps would be required to detect these proteins. We isolated liver and brain cell nuclei and extracted the nuclear pellet in a similar way as was done for the tissues. The 2-DE patterns showed that the nuclear extracts add a large number of new proteins

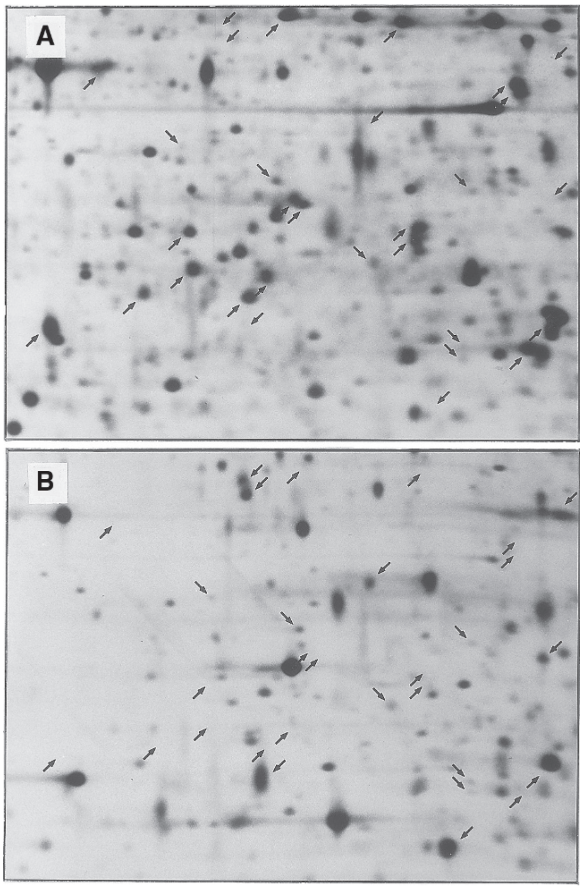

Fig. 3. Sections from 2-DE patterns shown in **Fig. 2.** The supernatant I + II (a) and the pellet extract (b) of liver are compared. (i) Some of the prominent protein spots present in the supernatant pattern (↗) but completely absent in the pellet extract pattern(↗), (ii) the reverse situation(↙), and (iii) some spots of low intensity present in both patterns(↘) are indicated. Other spots show a high intensity in one pattern but low intensity in the other. These spots may reflect naturally occurring unequal distributions of proteins between the two different fractions due to a formation of protein complexes rather than contaminations of one fraction by the other (*see* **Note 7**).

to those already known from the tissue extract patterns. However, protein spots present in both the nuclear and the tissue extracts occur as well, particularly in the acidic halves of the supernatant patterns. In general, the proteins represented by the SI+II, PE, and PS patterns can be considered as the main population of protein species of a tissue to which further species may be added by analyzing purified and concentrated subfractions. Therefore, the amount of spots detectable with fractionated extraction is larger than by total extracts but the effort involved is also exceptionally higher.

8. Explanation of correction factors used in **Table 2**: Factors were calculated to determine the amounts of urea, DTT-solution and ampholytes necessary to transmute any volume of a solution (theoretically water) into a mixture containing 9 *M* urea, 70 m*M* DTT and 2% ampholytes. Calculations of factors: 500 μL water (aqueous protein extract) + 540 mg urea (displaces 400 μL) + 50 μL DTT-solution (*see* **Subheading 2.2** item 5) + 50 μL ampholyte solution (commercial solutions which usually contain 40% ampholytes) = 1000 μL. If the volume of a protein solution to be mixed with urea, DTT and ampholytes is n μL, the amounts of the components to be added are: $(540 \div 500) \times n = 1.08 \times n$ mg urea, $(500 \div 50) \times n = 0.1 \times n$ μL DTT solution and $0.1 \times n$ μL ampholyte solution. If the protein solution already contains urea and DTT (*see* **Table 2**: PE preparation), the factor 0.0526 is used to calculate the ampholyte volume for this solution. Calculation of this factor: (500 μL extract + 400 μL urea + 50 μL DTT solution) ÷ 50 μL ampholyte solution = 0.0526. If a protein solution of n μL includes a cell pellet, i.e. insoluble material, n μL volume should be reduced by the volume of insoluble material (theoretically by the volume of the dry mass of this material). For this reason a pellet factor (e. g. 0.3) was introduced. This factor was determined experimentally using urea as an indicator. The factor reduces the volume n μL of a protein solution (containing a pellet) to volume n μL; n μL × 1.08 yields an amount of urea that is added to the n μL of the solution, at the border of solubility, i.e. about 9 *M*. Note that pellet III contains urea and DTT by the foregoing steps. Therefore, in this case the pellet volume was not taken into account when calculating the amounts for urea and DTT to be added to the final pellet suspension. In all these calculations no distinction was made between values measured in volumes (μL) and values measured in weights (mg). This makes the calculation somewhat incorrect but more practicable and reproducible. The inhibitor 2 solution was prepared as concentrated as possible to keep the volume of this solution small (1/50th of the homogenate volume, i.e. factor 0.01). This allows for ignoring the volume determination error that results when this inhibitor solution is added instead of including it to the total volume of the homogenate. The factor 0.08 for all inhibitor 1 (A, B and C) solutions was derived from the dilution requirements according to manufacturers instruction (CompleteTM tablets, ROCHE, Mannheim, Germany): 1 tablet should be dissolved in 2 mL buffer and this volume added to 25 mL of the homogenate, i.e. the volume of the inhibitor 1 solution to be added to n μL homogenate is $(2 \div 25) \times n = 0.08\,n$ μL. The volume of benzonase solution (ready-made solution from MERCK, Darmstadt, Germany) necessary to

digest DNA in the pellet III suspension was determined experimentally: a chromatin pellet was prepared from isolated liver cell nuclei and found to change from a gelatinous clot to a fluid if treated as follows: 1 g chromatin pellet + 1 mL buffer + 0.050 mL benzonase solution (= 0.025 mL Benzonase / mL homogenate), stirred for 30 min at 4°C. If benzonase from other sources is used the amount added has to be adjusted. Buffer factors used to calculate the volumes of buffer added to tissues or pellets are explained in the Introduction. Note that tissue to be homogenized must be free of any wash solutions otherwise the calculated buffer volumes will be too high.

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Plant Protein Sample Preparation for 2-DE

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1. Introduction

Most plant tissues are not a ready source for protein extraction and need specific precautions. The cell wall and the vacuole make up the majority of the cell mass, with the cytosol representing only 1 to 2 % of the total cell volume. Subsequently, plant tissues have a relatively low protein content compared to bacterial or animal tissues. The cell wall and the vacuole are associated with numerous substances responsible for irreproducible results such as proteolytic breakdown, streaking and charge heterogeneity. Most common interfering substances are phenolic compounds, proteolytic and oxidative enzymes, terpenes, pigments, organic acids, inhibitory ions, and carbohydrates.

Some species contain extremely high levels of interfering compounds. Banana (*Musa* spp.), for example, contains extremely high levels of oxidative enzymes (polyphenol oxidase) ***(1–3)*** and phenol compounds (simple phenols [dopa], flavonoids, condensed tannins, and lignin). Moreover, it contains high amounts of latex and carbohydrates ***(4)***. Phenolic compounds reversibly combine with proteins by hydrogen bonding and irreversibly by oxidation followed by covalent condensations ***(5)***, leading to charge heterogeneity and streaks in the gels. It is known that carbohydrates can block gel pores causing precipitation and extended focusing times, resulting in streaking and loss.

In the 1980s, much effort had been invested in the establishment of two-dimensional (2-D) gel electrophoresis sample preparation methods for plant tissue ***(6–8)***. The original 2-DE protocol consists of only one step, i.e., denaturing extraction in a lysis buffer ***(9)***. This one step protocol is restricted to “clean” samples and is rarely used for plant material. The majority of the plant protocols introduce a precipitation step to concentrate the proteins and to separate them from the interfering compounds. Proteins are usually precipitated by the addition

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of high concentrations of salts ***(10)***, extreme pH ***(11)***, organic solvents ***(12–14)*** or a combination of organic solvents and ions ***(15,16)***. Typical ions used for precipitation are ammonium sulfate and ammonium acetate. Ions effectively compete with water molecules on the protein surface, favoring protein-protein interactions over protein-solvent interactions and causing protein aggregation and precipitation. Organic solvents also decrease protein solubility by dehydrating the protein surface. They lower the solution dielectric constant, promoting charge-charge interactions between proteins. Typical organic solvents used for precipitation are methanol, ethanol, and acetone. Organic solvent precipitation is generally performed at low temperatures, since proteins are then less "soluble" ***(17)***. The protein precipitation step can be preceded by a denaturing or non-denaturing extraction step and is combined with one or two washing steps to remove introduced salt ions and other remaining interfering substances. The most commonly used method for extraction of plant proteins is the TCA/acetone precipitation method ***(15)***. Proteins are very sensitive to denaturation at low pH. Trichloroacetic acid (TCA) is a strong acid (pK_a 0.7) that is soluble in organic solvents. The extreme pH and negative charges of TCA together with the addition of acetone realizes an immediate denaturation of the protein along with precipitation, thereby arresting instantly the action of proteolytic enzymes ***(18)***. However, a disadvantage of TCA precipitated proteins is that they are difficult to redissolve ***(19)*** and the extreme low pH might create problems with basic chemical labeling methods. The development of succinimidyl ester derivatives of different cyanine fluorescent dyes that modify free amino groups of proteins prior to separation ***(20)*** was a major achievement for 2-DE in terms of reproducibility and throughput. The difference gel electrophoresis (DIGE) minimal labeling approach uses fluorophores that have a different absorption optimum, making it possible to run multiple samples simultaneously in the same gel. The different dyes were designed to assure that a protein acquires the same relative mobility irrespective of the dye used to tag them. The difference in MW introduced by the different length linkers is compensated by the different alkyl moieties opposite the linker moiety. Succinimidyl ester derivatives react with the nucleophilic primary amines, subsequently releasing the N-hydroxysuccinimide group. At a specific pH (8.5), these reagents react almost exclusively with the ε-amino group of lysine to form stable amide linkages that are highly resistant to hydrolysis. The pH of the protein solution is hence of utmost importance.

We have tested in the past different protocols to extract proteins from plant samples (***21***). In our hands, the phenol extraction protocol proved to be the most powerful. The protein precipitation step is in this method achieved by ammonium acetate and methanol and is preceded by a denaturing phenol extraction. We optimized this method for small amounts of banana fresh weight (***21***) and lyophilized tissues (***22***). This "micro" phenol protocol is applicable for a wide variety of plant species since we successfully applied it to apple, banana, pear *(23)*, potato and stevia. We describe here in detail this successful "micro" phenol extraction protocol.

2. Materials

1. All the materials that were used were clean and the chemicals are of the highest purity in order to not interfere with the electrophoresis process and with the mass spectrometry.
2. DTT (dithiothreitol) has a limited shelf life and should not be added to the different stock solutions. Make always a fresh 10% (w/v) solution and add prior to use.
3. All consumables were purchased from Acros Organics (Geel, BE) unless stated otherwise.

2.1. Protein Extraction

1. Liquid nitrogen.
2. Pestle and mortar (washed and baked in an oven at 180°C overnight prior to use).
3. Phenol, acetone and methanol compatible tubes and pipettes.
4. 10 % (w/v) DTT (GE Healthcare, Diegem, BE). Make freshly in MQ H_2O.
5. Extraction buffer: 50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% (w/v) DTT, 30% (w/v) sucrose and complete protease inhibitor cocktail (according to the manufacturer, Roche Applied Science) in MQ H_2O. Prepare freshly (*see* **Note 1**).
6. Tris buffered phenol (pH 8.0) (*see* **Notes 2** and **3**).
7. Precipitation buffer: 100 mM ammonium acetate in methanol. Store at −20°C.
8. Cold acetone. Store at −20°C.
9. Lysis buffer: 7 M urea (GE Healthcare, Diegem, BE), 2 M thiourea (Fluka, Bornem, BE), 4% (w/v) CHAPS (GE Healthcare), 0.8% (v/v) IPG-buffer (GE Healthcare), 1% (v/v) DTT (GE Healthcare) and 30mM Tris in MQ H_2O. Store aliquots at −80°C (*see* **Notes 4** and **5**).
10. Protein Quantification kit 2D Quant (GE Healthcare).

2.2. Protein Separation

1. 1% (w/v) bromophenol blue: bromophenol blue (GE Healthcare) and 50 m*M* Tris-base in MQ H_2O.
2. Rehydration buffer: 6 M urea, 2 M thiourea, 0.5% (w/v) CHAPS, 10% (v/v) glycerol, 0.002% (v/v) bromophenol blue, 0.5% (v/v) IPG-buffer and 0.28% (v/v) DTT in MQ H_2O (*see* **Note 6**).
3. 24 cm IPG-strips pI 4-7 (GE Healthcare) (*see* **Note 7**).
4. Reswelling tray (GE Healthcare).
5. Isoelectric focusing apparatus IPGphor II (GE Healthcare).
6. Sample cups (GE Healthcare).
7. Ceramic 12-strip sample tray (GE Healthcare).
8. Filter paper electrode pads (GE Healthcare).
9. Mineral cover oil (GE Healthcare).

10. Gel casting chamber (GE Healthcare).
11. SDS-PAGE apparatus Ettan Dalt 6 (GE Healthcare).
12. Cooling bath type 1140S (VWR international, Leuven, BE).
13. Power supply Power PAC 3000 (Bio-Rad, Nazareth, BE).
14. Equilibration buffer I: 6M urea, 30% (v/v) glycerol, 2% (v/v) SDS, 0.002% (v/v) bromophenol blue, 50mM Tris-HCl pH 8.8 and 1% (w/v) DTT in MQ H_2O. Store aliquots at −20°C.
15. Equilibration buffer II: 6M urea, 30% (v/v) glycerol, 2% (v/v) SDS, 0.002% (v/v) bromophenol blue, 50mM Tris-HCl pH 8.8 and 4.5% (w/v) iodoacetamide in MQ H_2O. Store aliquots at −20°C (*see* **Note 8**).
16. SDS 10%. Store at room temperature (*see* **Note 9**).
17. Laemmli running buffer: 25mM Tris, 0.1% (v/v) SDS, 192mM glycine in demineralized water. Do not adjust the pH. Store a 10x buffer at room temperature.
18. Agarose sealing solution: 0.5% (w/v) agarose (ultra pure, GibcoBRL, Merelbeke, BE) 0.002% (v/v) bromophenol blue in Laemmli running buffer.
19. 10% (w/v) Ammonium persulfate. Make freshl in MQ H_2O.
20. Thirty percent acrylamide/bis solution (37.5:1 with 2.6% C) (Bio-Rad). Store at 4°C (*see* **Note 10**).
21. N,N,N,N -Tetramethyl-ethylenediamine (TEMED) (Bio-Rad). Store at room temperature (*see* **Note 11**).
22. Tris 1.5M pH 8.8 in MQ H_2O. Store at 4°C.
23. Water-saturated isobutanol. Shake equal volumes of MQ H_2O and isobutanol and allow separation. Use the top layer. Store at room temperature.

2.3. Protein Labeling and Scanning

1. 10m*M* lysine.
2. CyDye DIGE fluors (Cy2, Cy3 and Cy5) (GE Healthcare). Store at −20°C.
3. N,N-dimethylformide (DMF). Store at room temperature (*see* **Note 12**).
4. Typhoon™ imager (GE Healthcare).
5. Low fluorescent glass plates (GE Healthcare).

3. Methods

3.1. Protein Extraction

1. Transfer fresh plant tissue to a liquid nitrogen pre-cooled mortar and grind in liquid nitrogen (*see* **Note 13**).
2. Transfer 50 to 100mg of frozen tissue powder to an extraction tube (2mL), add 500µL extraction buffer and vortex 30sec (*see* **Note 14**).
3. Add 500µL of buffered phenol and vortex 10min at 4°C.
4. Centrifuge for 3min, 6000g at 4°C, collect the upper phase, i.e., the phenolic phase, transfer to a new tube and discard the lower phase (*see* **Note 15**).
5. Re-extract by adding 500µL of new extraction buffer.
6. Centrifuge for 3min, 6000g at 4°C.
7. Transfer the phenolic phase into a new 2mL tube and precipitate the proteins overnight (or at least 2.5 h) with 5 volumes 100m*M* ammonium acetate in methanol at −20°C.

8. Centrifuge 60 min, 16 000 g, at 4°C.
9. Remove the supernatant and rinse the pellet twice (do not resuspend) in 2 mL rinsing solution (cold acetone/0.2 % DTT). After the first rinse leave in rinsing solution for 1 hour at −20° C. After rinsing, centrifuge 30 min, 13 000 rpm, at 4°C.
10. Dry the pellet (*see* **Note 16**).
11. Suspend the pellet in 100 mL lysis buffer (optimum concentration is 1–5 µg/µL) (*see* **Note 17**).
12. Clear samples by centrifugation (16 000 g, 2 × 30 min at 18° C) (*see* **Notes 18** and **19**).
13. Quantify the samples (*see* **Note 20**).

3.2. Protein Labeling

1. Briefly spin the fluor stock solutions.
2. To 4.2 µL DMF add 2.8 µL DMF reconstituted fluor stock solution (1 nmol/ µL) in a microfuge tube (200 µL). Ensure all fluor is removed from the pipette tip by pipetting up and down. This is the working fluor sample solution.
3. Add 50 µg of protein sample (without DTT and IPG buffer) to a microfuge tube (200 µL) and add 1 µL of working fluor sample solution.
4. Mix fluor and protein sample thoroughly and leave on ice for 30 min in the dark.
5. Repeat this for all samples (*see* **Note 21**).
6. Add 1 µL of 10 mM lysine to stop the reaction. Mix and leave for 10 min on ice in the dark. The labeled samples can be stored for at least 3 months at −80°C.
7. Dilute the samples with an equal volume of Lysis Buffer (with 2x DTT and 2x IPG buffer) and leave at room temperature for 10 min.

3.3. Protein Separation: First Dimension

1. Rehydrate IPG dry strips with rehydration buffer in a reswelling tray for at least 8 h and cover with mineral oil.
2. Apply the rehydrated IPG gel strips onto the cup-loading tray, gel side upward and acidic ends facing toward the anode.
3. Moisten two filter paper electrode pads with demi water and apply the moistened filter paper pads on the surface IPG gel covering the anodic and cathodic ends of the IPG strip.
4. Position the movable electrodes at the extremes of the electrode filter paper pads.
5. Overlay the IPG strips (and empty lanes) with several mL of cover fluid (120 mL) (*see* **Note 22**).
6. Position the movable sample cup either near the anode, and gently press the sample cup onto the surface of the IPG gel strip (*see* **Notes 23** and **24**).
7. Dilute your sample in rehydration buffer (final volume 150 µL) and pipette the sample into the cup.
8. Program the instrument (desired volthours, voltage gradient, temperature, etc.) and run IEF according to the settings recommended in **Table 1**. Current and power settings should be limited to 0.05 mA and 0.2 W per IPG gel strip, respectively. Optimum focusing temperature is 20°C.
9. After IEF, IPG gel strips can be stored between two sheets of plastic film at −78°C up to 3 months.

Table 1
Program for Cup Loading IPGphorII

Step	Mode	Voltage	
1	Step and hold	300	3h
2	Gradient	1000	6h
3	Gradient	8000	3h
4	Step and hold	8000	32 kVh

3.4. Protein Separation: Second Dimension

1. Cast the polyacrylamide gels. For 600 mL (6 gels) 12.5% acrylamide gels: add 250 mL 30% acryl/bisacryl, 150 mL Tris buffer pH8.8, (1.5 M), 187 mL MQ H_2O, 300 µL TEMED, 6 mL 10 % SDS and 2.4 mL 10% APS, under continuous stirring (*see* **Note 25**).
2. Cover with water saturated isobutanol and cover to prevent drying out.
3. Allow polymerization overnight (*see* **Note 26**).
4. Remove the isobutanol prior to use by rinsing with MQ (*see* **Note 27**).
5. Place the IPG strips in an individual tube and add 7 mL of equilibration buffer I. Shake for 15 min.
6. Remove the first equilibration solution and replace by 7 mL of equilibration buffer II. Shake for 15 min.
7. Prepare the running buffers for upper (2x) and lower chamber (1x) and fill the lower chamber.
8. Rinse the strips with running buffer.
9. Place the gel cassettes in an upright position and apply the strips and fill with 2–3 mL of hot (60°C) 0.5 % agarose solution. Make sure that the strip is in close contact with the second dimension gel without air bubbles.
10. Place the cassettes in the running tank, place the upper buffer chamber and fill it further with running buffer.
11. Run the gels at 2 W per gel overnight.

3.5. Scanning

1. Prescan the gels at the 3 wavelengths at a low resolution and adjust the PMT (photomultiplier tube) to ensure a maximum pixel intensity between 70,000 and 100,000 pixels using a Typhoon™ imager (GE Healthcare).
2. Scan the gels at the optimal pixel intensity. See **Fig. 1** for a representative DIGE gel. A protein spot map realized in ***(24)*** containing MS/MS identified spots can be found at http://www.pdata.ua.ac.be/musa/

4. Notes

1. Our extraction buffer has been designed to minimize enzymatic reactions and to remove as much interfering compounds as possible. Sucrose is added to the buffer to create a phase inversion. The buffer forms the aqueous

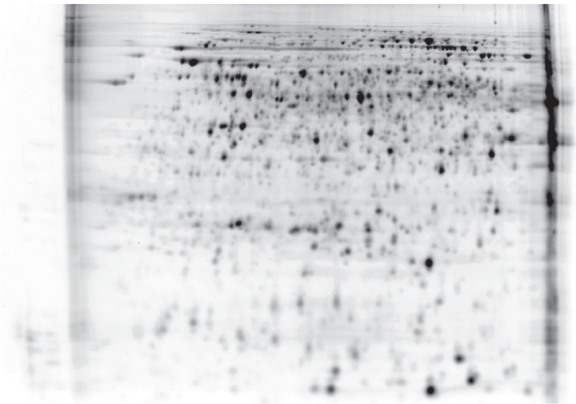

Fig. 1. Representative gel of the banana meristem proteome labelled with Cy3. 24 cm IPG strips pI 4–7.

lower phase containing carbohydrates, nucleic acids and cell debris and the upper phenol rich phase contains cytosolic and membrane proteins, lipids, and pigments. The high pH of the buffer inhibits most common proteases, assures that the abundant phenolic compounds are mainly ionized (inhibiting H bonding with the proteins) and neutralizes acids that are released by disrupted vacuoles. KCl facilitates the extraction of proteins (salting in effect) and EDTA inhibits metalloproteases and polyphenol oxidase by chelating the metal ions. DTT is a powerful reducing agent that does not actually prevent the oxidation of plant (poly)-phenols (quinones) but reduces them to form thio-ethers. The protease inhibitor cocktail is added to inhibit proteases that are released upon cell rupture.

2. The pH of the phenol is important. We opted to buffer the phenol to pH 8.0 instead of water buffered phenol ***(25–27)*** to assure that the interfering nucleic acids are partitioned to the buffer phase and not to the phenol rich phase.
3. Phenol is toxic and needs to be handled with great care. Wear gloves and work in a dedicated room. Use only under the hood and make sure that the waste (tips, pipettes, tubes) is disposed safely.
4. Tris is added to the lysis buffer to get the pH stable between 8 and 9 to assure proper labeling and to avoid pH adjusting with other salts afterward since salts affect the electrophoresis process. If the proteins are visualized with a post staining procedure the Tris can be omitted.
5. DTT and IPG-buffer are added freshly prior to use. For the DIGE labeling they must be added after labeling since they are subject to labeling.
6. Glycerol is added to the buffer in order to obtain an even reswelling of the strips and to minimize electro-osmotic flow (counter flow of water ions).

7. The size of the strips plays an important role in the resolution of the gel. The longer the strip, the better the resolution.
8. As for the DTT, the iodoacetamide is not added to the stock solution but prior to use.
9. SDS is irritating. The powder should be handled with care since it can be inhaled very easily and should always be weighted under a hood. Make always a stock solution of 10% (w/v).
10. Acrylamide is a neurotoxin when unpolymerized and so care should be taken not to receive exposure. Wear gloves, work under a hood in a dedicated room.
11. TEMED has a limited shelf life. Buy therefore small amounts. The quality is important for a good polymerization.
12. The DMF must be high quality anhydrous (specification: 0.005% H_2O, ≥99.8% pure) and every effort should be taken to ensure it is not contaminated with water. DMF, once opened will start to degrade to produce amine compounds. Those amines will react with the succinimidyl ester derivatives of the cyanine dyes, reducing the concentration of dye available for protein labeling. DMF is toxic and any contact should be avoided. Wear gloves.
13. The grinding process is very important. Do not stop grinding until the power is very fine and homogeneous.
14. It is important to keep this period as short as possible in order to keep enzymatic reactions like oxidation of polyphenols and protease activity to the strict minimum. All enzymes are considered to be irreversibly inactivated after adding phenol as is the case with TCA precipitation.
15. Take care to avoid transferring parts of the lower phase and the inter phase since they contain a lot of interfering compounds.
16. The drying of the pellet is very important. Take care that the pellet is dry enough (indicated by a slight color change) but not over dry. A pellet that is too dry will often be very difficult to resuspend. It is recommended to dry the pellet under a hood and to follow up the drying process.
17. A recalcitrant (difficult-to-redissolve) pellet can elegantly be forced to redissolve by freezing the solution at −20°C. The formed crystals will break the pellet upon vortexing. Repeated freezing and thawing of the sample must be avoided!
18. A lower temperature of the centrifuge can result in crystallization of urea.
19. Storage in a freezer at −78°C is preferred. Repeated freezing and thawing of the sample must be avoided. Make aliquots of the desired quantity and thaw only once!
20. The quantification of the proteins is very important but challenging. Each quantification method has its limitations since the concentration of a complicated protein mixture is estimated based on the quantification of one

reference protein usually BSA. It is therefore important to have sufficient replications and to stay within the optimal linear range of the reference protein. Each sample has to be equally labeled and loaded.

21. The power of the DIGE approach arises from the use of an internal standard, which is a common representative mixture of all analyzed samples. The internal standard is a mixture of an equal amount of all analyzed samples and is labeled with Cy2. This internal standard is used to calculate a standardized abundance of each spot and to match the spots across the gels. In order to anticipate any dye specific effect, the samples should be labeled at random with Cy3 and Cy5. All pre-culture sample points should be randomized over the gels to anticipate gel-specific effects.
22. It is very important that all the strips are covered. Uncovered strips will dry out and urea will start to crystallize. If less than 12 strips are used also the remaining lanes should be filled to prevent crystallization. The oil can move from one lane to the other.
23. Cup loading involves applying the samples at a specific experimentally determined zone of a rehydrated strip. All proteins approach their pI only from the same side. However, the application point of the cup should be carefully chosen. Proteins tend to precipitate at their pI and can lead to aggregation at the sample entry point. Therefore, the placement of the cup is sample specific and depends on the pI range of the IEF-strip. The cup is placed at the cathodal side in case of an acidic zoom strip and at the anodal side in case of a basic zoom strip.
24. It is very important that the cups do not leak. Leakage can be checked by adding a small volume of rehydration buffer if desired.
25. In order to better control the start of the polymerization reaction use acrylamide, water and Tris that is stored at 4°C.
26. Although the gels look polymerized after a few hours, there is still silent polymerization going on. For reproducible gels, it is therefore very important to perform the polymerization overnight and always at the same room temperature.
27. The shelf life of the Laemmli buffered gels is limited and the quality is guaranteed only for a few days.

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14

Preparation of Bacterial Samples for 2-D PAGE

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1. Introduction

Sample preparation is a very crucial step in two-dimensional (2-D) gel electrophoresis in which the proteins of the sample are brought into a state where they can be separated by isoelectric focusing in the first dimension. The proteins must be denatured, reduced, and solubilized, and they must be kept so during electrophoresis without changing their pI. The buffer for this purpose is traditionally called the lysis buffer.

Most bacterial studies aim to solubilize as many proteins as possible to obtain the best possible representation of the total protein content or protein expression under the investigated biological circumstances. However, prefractionation, or the successive application of different chemical reagents, can be used to investigate proteins with certain characteristics. Note that the gels will reflect the proteome of the bacteria at the time the proteins are solubilized and that preceding centrifugations or other manipulations may stress the bacteria, thereby influencing the protein profile.

The solubilization procedure is highly dependent on the nature of the sample. Some bacteria are readily lysed by the constituents of the lysis buffer. Others must be disrupted mechanically and, for some, it may be necessary to remove the cell wall by enzymatic digestion prior to mechanical disruption. **Subheading 3.1** provides a general protocol for solubilization. All reagents are described in the notes together with common alternatives.

Some samples may contain nonprotein substances in amounts that are incompatible with especially first dimensional electrophoresis and, thus, have to be removed from the sample. Salts and most other compounds can be removed by protein precipitation, as described in **Subheading 3.2.1**. If nucleic acids are present in high amounts, these may have to be removed by enzymatic digestion, which is described in **Subheading 3.2.2**.

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In addition, highly abundant proteins may cause problems for 2-D PAGE by preventing optimum focusing in the first dimension or by masking large areas of the gel. In such cases, it may be necessary to carry out prefractionations or to specifically remove the abundant proteins by immunoprecipitation. A novel method of enrichment as a result of low abundant proteins is the ProteoMiner technology from BioRad. By the use of a peptide labrary coupled to column material, the amount of highly abundant proteins is decreased because of binding site saturation and, by passing large volumes of sample over the column, the relative amount of low abundant proteins is simultaneously increased. Prefractionation can be obtained by various means such as chemical extractions or chromatographic or electrophoretic techniques ***(1)***, but these methods fall beyond the scope of this chapter.

All procedures should be kept as simple as possible to ensure reproducibility and because proteolytic degradation must be considered a risk. When bacteria are disrupted, proteases will be released and degrade the proteins of the sample, if not inhibited. As folded proteins are less susceptible to proteolysis than denatured proteins and as proteases are often more resistant to denaturation than most other proteins, solubilization in urea will often make the problem worse. However, most proteases will be inactivated by disruption in lysis buffer containing thiourea, in 2% SDS or in precipitation solution containing 10% TCA. Still, all handling of the sample after disruption of the bacteria should be carried out as quickly as possible and on ice to minimize proteolytic degradation. It may also be necessary to add protease inhibitors.

Several reagents used for the sample preparation are toxic and/or carcinogenic. For safety reasons use protective gloves and glasses and work in a fume hood.

2. Materials

1. Lysis buffer (*see* **Note 1**): 7 M Urea (2.10 g) (*see* **Note 2**), 2 M thiourea (0.76 g) (*see* **Note 3**), 65 mM DTE (650 µL of a 0.5 M stock) (*see* **Note 4**), 4% (W/V) CHAPS (0.2 g) (*see* **Note 5**), 2% (v/v) Pharmalyte pH 3–10 (*see* **Note 6**), 40 mM Tris base (*see* **Note 7**), a trace of bromphenol blue (*see* **Note 8**).
2. Presolubilization solution: 2% SDS, 65 mM DTE.
3. Precipitation solution: 10% Trichloracetic acid (TCA) in acetone, 20 m*M* DTE.
4. DNase/RNase solution: 1 mg/mL DNase I, 0.25 mg/mL RNase A, 50 m*M* $MgCl_2$.

3. Methods

3.1. General Solubilization Protocol

It is crucial that all bacteria are disrupted so that the lysis buffer gains access to all proteins. In studies where multiple extractions with different chemical reagents are employed sequentially, it will mask the result if more and more bacteria are disrupted during the procedure. The best method for disruption is dependent on the type of bacteria. In most cases, disruption by sonication will do, but it may be necessary to add lysosyme to break down cell walls. It is advantageous to perform the disruption in SDS or in lysis buffer containing thiourea in which most proteases are denatured *(2)*. If prolonged manipulation, such as fractionation by different steps of centrifugation, must be performed, protease inhibitors should be added (*see* **Note 9**). In the procedure described below, the bacteria are sonicated in 2% SDS, 65 mM DTE and boiled to enhance the protein solubilization in general *(3)*. It has been suggested *(4)* that SDS used for presolubilization does not interfere with first dimensional electrophoretic separation because it forms micelles with the nonionic detergent of the lysis buffer and migrates out of the strip. Still, the amount of SDS should be kept low compared to the amount of detergent in the lysis buffer *(5)* (*see* **Note 10**). **Figure 1** shows a silver stained gel loaded with 100 µg of *Chlamydia pneumoniae* protein that was presolubilized by boiling in

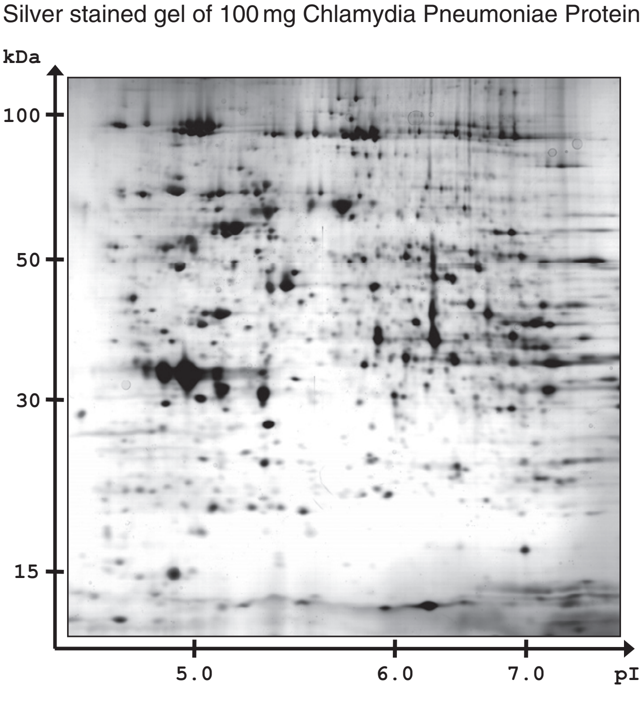

Fig. 1. A pellet of purified *Chlamydia pneumoniae* elementary bodies (12) was resuspended in 1% SDS, 50mM Tris-HCl pH 7.0, sonicated and boiled for 5 min. Cooled sample was diluted 1:10 in lysis buffer, sonicated briefly, left at room temperature for one hour and centrifuged at 20.000 x g for 15 minutes. 350 µL of the supernatant containing 100 µg protein was loaded onto a pH 3–10 non-linear immobilized pH gradient strip (Amersham Biosciences) and focused for 120.000 Volt hours. Second dimension was 9–16% linear gradient SDS PAGE. The gel was silver stained.

1% SDS and 50mM Tris-HCl and then diluting it in the described lysis buffer to a final concentration of 0.1% SDS (*see* **Note 11**).

The protocol below describes the solubilization of proteins from pelleted bacteria. If proteins have been precipitated, for example, for desalting, the lysis buffer should be added directly **(step 8)** in the highest possible amount (*see* **Note 12**).

1. Start out with an appropriate amount of bacteria as pellet (*see* **Note 13**).
2. Add four times the pellet volume of 2% SDS, 65m*M* DTE (*see* **Note 14**).
3. Sonicate three times for 2–20 sec depending on sample size (*see* **Note 15**).
4. Spin shortly to collect the sample (*see* **Note 16**).
5. Resuspend any pellet that may have formed.
6. Boil for five min (*see* **Note 17**).
7. Allow the sample to cool
8. Add 8 volumes of lysis buffer to one volume of extract (*see* **Note 18**).
9. Sonicate three times for 5 sec, cool between sonications (*see* **Note 19**).
10. Leave the sample on a rocking table for 30 min.
11. Spin at 20,000 × g for 15 min and collect the supernatant (*see* **Note 20**).
12. Asses the protein concentration (*see* **Note 21**).
13. Run first dimension immediately or store the sample at −70°C for several months (*see* **Note 22**).

3.2. Sample Purification

Common contaminants in 2-D PAGE studies are salts, small ionic compounds, polysaccharides, nucleic acids and lipids. Salt is the most likely reason if bad first dimensional focusing is observed. Enhanced conductivity and water migration in the strip due to high concentrations of salts will cause horizontal streaks. The concentration of salt should preferably be below 10 m*M* when samples are loaded by strip rehydration. Small charged substances may likewise disturb the isoelectric focusing, especially at the acidic end, as such are often negatively charged. Polysaccharides may clog the gel of the strip and may complex proteins by electrostatic interactions. Lipids may also clog the gel but are mainly a problem due to complexing of hydrophobic proteins and binding of detergent. Nucleic acids may clog the gel, bind proteins through electrostatic interactions, and cause streaking in itself, especially in silver staining.

Buffer shift using spin of drip columns, dialysis, and precipitation **(Subheading 3.2.1)** are straightforward and effective ways to reduce the concentration of salt and small ionic compounds to an acceptable level. Dialysis causes a minimal loss of sample, but requires relative large volumes of solute and is rather time consuming. Spin dialysis using, for instance, Amicon Ultra from Millipore is faster and requires no extra volume of solute, but protein may be lost by adsorption onto the dialysis membrane. Similar issues exist for buffer shifting. Precipitation may also be used to remove polysaccharides and, to some extent, lipids. Large polysaccharides can be removed by ultracentrifugation. If lipids are causing major problems, the amount and nature of detergent must be optimized for the particular sample. High amounts of nucleic acids may require treatment with DNase/RNase **(Subheading 3.2.2)**.

The presence of proteases is likely to be a problem during sample purification and in such case protease inhibitors must be added (*see* **Note 9**). It must be stressed that sample purification preceding addition of lysis buffer should only be carried out if necessary and not as a standard part of the sample preparation.

3.2.1. Precipitation

Precipitation is very efficient for removal of most contaminants including salts, but no precipitant will precipitate all proteins, and some proteins will be difficult to resuspend following precipitation. This is a particular problem when a picture of the total protein content is desired.

A combination of TCA and acetone is the most common precipitant in 2-D PAGE studies, as it is more effective than either of these reagents alone. Besides, very few proteases are active in 10% TCA. Resolubilization is easier after precipitation with acetone alone (75% final concentration), but precipitation wil not be as efficient as with TCA. The TCA/acetone precipitation described here is essentially as in ***(6)***.

1. Add 10% TCA in ice-cold acetone with 20mM DTE to the sample (*see* **Note 23**).
2. Leave at −20°C for two hours (*see* **Note 24**).
3. Centrifuge at 10.000 × g for 10 min.
4. Wash with cold acetone containing 20m*M* DTE.
5. Repeat wash.
6. Let the pellet dry to remove residual acetone.
7. Resuspend pellet in lysis buffer (*see* **Subheading 3.1**).

3.2.2. DNase/RNase Treatment

If nucleic acids are present in high amounts, the sample will appear viscous and a smear will be seen after silver staining. If ultracentrifugation does not solve the problem, enzymatic digestion will.

1. Add 1/10 of the sample volume of a solution containing 1 mg/mL DNase I, 0.25 mg/mL RNase A and 50 mM $MgCl_2$ (*see* **Note 25**).
2. Incubate on ice for 20 min.

4. Notes

1. Absolute amounts are to make 5 mL. All reagents must be analytical grade. Use doubly distilled water. The solution is best mixed in a 10 mL tube on a rotating device. The solution should be made fresh before use or alterna-

tively frozen in aliquots at −70°C and only thawed once. The solution must not be heated above 37°C.

The composition of the lysis buffer is essential for the final result of 2-D PAGE and different lysis buffers may be optimum for different samples and for different proteins in one sample. The function of the lysis buffer described here is to bring as many proteins in the sample as possible into solution and keep them in solution during electrophoresis. As isoelectric focusing is best carried out under denaturing and reducing conditions, the lysis buffer should solubilize, denature, and reduce the proteins of the sample. At the same time, the lysis buffer must not change the pI of the proteins, and it must not be highly conductive. Thus, uncharged components are preferred. Most lysis buffers are still based on that introduced by O'Farrel in 1975 ***(4)*** containing urea as denaturing agent, a detergent, a reducing agent and carrier ampholytes. The standard lysis buffer described here is based on ***(3)*** and the characteristics of each reagent are described in the following notes.

2. Urea, $(NH_2)_2CO$, is a non-charged chaotrope that disrupts non-covalent bonds and thereby denatures proteins. It is the main denaturant in all lysis buffers used in 2-D PAGE and it can be brought into solution in concentrations up to 9.8 M if no thiourea is added. Urea in solution is in equilibrium with ammonium cyanate, which in the form of isocyanic acid will react with amino groups of lysine and arginine residues and the amino terminus of proteins causing carbamylation. The carbamylation of an amine group removes a positive charge from the protein causing a shift toward the acidic side in the gel. Furthermore, it prevents N-terminal sequencing and some enzymatic digests. To avoid carbamylation use only freshly prepared urea solutions. A urea solution should not be left at room temperature for longer periods and should never be heated above 37°C ***(7)***.
3. Thiourea, $(NH_2)_2CS$, improves the solubilization of especially hydrophobic proteins during first dimension ***(8)*** and, in combination with urea, it can be used in concentration up to 2.5 M. The addition of thiourea reduces the solubility of urea and combinations of 7 M urea and 2 M thiourea or 8 M urea and 0.5 M thiourea are most common. The addition of thiourea to the lysis buffer has a pronounced inhibitory effect on proteases, which may still be active in high concentrations of urea alone ***(2)***. As thiourea can hinder the binding of SDS to proteins, it should not be included in the buffers used to equilibrate strips prior to second dimension ***(8)***.
4. DTE, dithioerythritol, MW: 154.3, make a 0.5 M stock solution and store at −20°C. DTE has the same strong reducing power as dithiothreitol (DTT) and both can be used in concentrations from 10–100 m*M*. At alkaline pH both DTE and DTT are charged and migrate toward the anode during first dimen-

sion, which may leave the basic end of the strip without reducing agent and thus cause streaking as a result of reoxidation and precipitation. An alternative and very strong reducing agent is tributyl phosphine (TBP). It can be used in concentrations as low as 2 m*M* and is noncharged, which means that it keeps all proteins reduced throughout the first dimension thereby enhancing the resolution ***(9)***. TBP is stable, but spontaneously inflammable in air. Make a 200m*M* stock in anhydrous isopropanol and store under nitrogen at 4°C ***(9)***. Other alternatives are to alkylate the cysteines prior to first dimension or to oxidize the disulfides by performing the isoelectric focusing in the presence of excess hydroxyethyldisulphide (HED), which is commercially available as DeStreak (GE Healthcare) and includes detailed protocols for its use.

5. CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, is a zwitterionic detergent that is used in many 2-D PAGE studies. Zwitterionic or nonionic detergents are preferred to anionic detergents like SDS that interferes with the isoelectric focusing in first dimension (*see* **Note 1**). The efficiency of many zwitterionic detergents has been investigated and sulfobetaines with a hydrophobic tail of 12-16 alkyl carbons and an empirically determined linker in between have been found to be good alternatives to CHAPS and superior for some samples ***(10)***. Which detergent is best for a given sample can still not be predicted. The zwitterionic agent ASB-14 ($C_{22}H_{46}N_2O_4S$, Calbiochem) with a 14-carbon alkyl tail, the nonionic Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$ with an average number (n) of ethylene oxide of 9 to 10) or the maltoside n-Dodecyl β-D-maltoside ($C_{24}H_{46}O_{11}$, Sigma-Aldrich) would be good first-choice alternatives if CHAPS does not give satisfactory results ***(11)***.
6. Pharmalyte 3–10 can be used for most immobilized pH gradient strips, but if narrow strips or very basic strips are used, carrier ampholytes that match the pH range of the strip should be chosen. Ask the strip supplier if in doubt. Pharmalyte 3–10 is a mixture of carrier ampholytes with pI between 3 and 10. These are small amphoteric compounds with a molecular weight below 1 kDa that have a high buffering capacity at their pI but do not bind proteins due to their high hydrophilicity. When we use 2% v/v of Pharmalyte 3–10 (Amersham Pharmacia) it gives a final concentration of carrier ampholytes of 0.72% in the lysis buffer since "Pharmalyte 3-10" is 36% (w/v). Carrier ampholytes help keep proteins in solution during first dimension and especially prevent hydrophobic interactions between proteins and the IPG in the basic end of the strip. Furthermore, the precipitation of nucleic acids is improved by carrier ampholytes.
7. Tris base is added to raise pH of the lysis buffer to 8.5. Without the addition of base, pH of the lysis buffer would be about 5.5. At alkaline pH more proteins will be anionic and thus not bind to DNA. However, the pH for

optimal solubilization will vary among samples, and Tris base is left out in many studies. Note that if the sample is to be labbeled before electrophoresis, pH may be improtant for the labelling reaction, which for instance is the case for CyDye labelling in the DIGE (GE Healtcare) approach.

8. Bromphenol blue should be added in a small amount to color the solution lightly blue. The color will move toward the anode during first dimension, which can be used to check that the isoelectric focusing is ongoing. However, the color will disappear before the first dimension is finished and cannot be used as an indicative of when to stop.
9. Protease inhibitor cocktails are available from several commercial laboratory reagent suppliers but most proteases will be inhibited by adding 2 m*M* EDTA, 1 mM PMSF, 1 µM Pepstatin A, and 13 µM Bestatin. EDTA chelates free metal ions, thereby inhibiting metalloproteases, make a 0.5 M stock solution in water, pH 8.0. PMSF (phenylmethylsulfonyl fluoride), inhibits serine proteases and some cysteine proteases, make a 100 mM stock solution in methanol. Pepstatin A inhibits aspartic proteases, make a 1 mM stock stock solution in methanol. Bestatin inhibits aminopeptidases, make a 13 mM stock solution in water.
10. When NP-40 is used as detergent in the lysis buffer it has been reported ***(5)*** that the ratio of NP-40 to SDS should be at least 8 to avoid streaking. NP-40 (Nonidet P-40) is a nonionic detergent that is very similar to Triton X-100 and the properties are often reported as being identical. NP-40 (Roche) is $C_{15}H_{24}O(C_2H_4O)n$, where $n = 9–10$ on average.
11. No reducing agent was added during presolubilization.
12. If the sample is applied by strip rehydration of 18 cm strips, the maximum amount is 350 µL per strip.
13. For most bacteria 25–100 µg of protein is appropriate for silver staining, 100–150 µg for Western blotting, and 0.5–2 mg for preparative gels, when 18 cm immobilized pH gradient strips in the pH range of 3–10 or similar broad range intervals are used.
14. Be sure not to add more SDS than it can be diluted to 0.25% in lysis buffer. If all the sample is to be used for one gel using 350 µL lysis buffer to rehydrate an immobilized pH gradient strip, this means that no more than 40 µL of 2% SDS should be added. However, if streaking is observed, try lowering the amount of SDS used for presolubilization. For some samples pH must be buffered to optimize solubilization. Use for instance 50–100 m*M* Tris-HCl, pH 7.0. The optimal pH may vary from sample to sample. Be aware that the final concentration of salt should not exceed 10 m*M* when samples are loaded by strip rehydration (*see* **Subheading 3.2**).
15. Adjust the amplitude of the sonicator so that microbubbles are formed, and keep the tip of the probe deep in the sample to avoid too much foam formation. The sample should be cooled between sonications.

16. It cannot be avoided that some foam is formed during sonication and this should be spun down before the sample is boiled, in order to avoid protein coagulation in drying bubbles.
17. DTE develops toxic gas upon heating. Boil in a fume hood.
18. Dilute the sample as much as possible in lysis buffer (*see* **Notes 13** and **14**).
19. When the sample is in lysis buffer containing urea it is important to keep the temperature below 37°C in order to avoid carbamylation of proteins. If boiling in SDS is left out, the sonication may have to be extended.
20. The centrifugation step is important to remove cell debris and precipitated DNA and it should not be left out.
21. Several constituents of the lysis buffer may cause problems for assessment of protein concentration. Carrier ampholytes, CHAPS, and other detergents will bind most dyes and reduction of cupric ion cannot be employed in the presence of thiourea and DTE. Thus, the protein must be selectively precipitated and then measured. This may be done with the 2-D Quant Kit form Amersham Pharmacia. Alternatively, the protein may be estimated in a parallel sample which is not solubilized in lysis buffer. This may not give the actual protein concentration in the lysis buffer but will in most cases provide adequate information to determine the load.
22. Repeated freeze thaw cycles should be avoided due to the risk of carbamylation and because the solubility of some proteins may be changed by the process.
23. The bacteria must be disrupted beforehand by an appropriate method (*see* **Subheading 3.1**). If the bacteria are disrupted directly in the precipitation buffer most proteases will be inactivated by the TCA.
24. The precipitation time should not be longer than the minimal time required for satisfactory precipitation. For some samples 15 min will do while others must be incubated overnight. Be aware that prolonged exposure to the very acidic solution may cause protein degradation.
25. The bacteria must be disrupted beforehand by an appropriate method (*see* **Subheading 3.1**). If active proteases are present it may be necessary to add protease inhibitors (*see* **Note 9**) even if the DNase/RNase treatment is carried out on ice.

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Preparation of Bodily Fluids for 2-D PAGE

Anthony G. Sullivan, Henry Brzeski, Jeban Ganesalingam, and Manuel Mayr

1. Introduction

The importance of bodily fluids as a source of clinically relevant biomarkers/surrogate markers of human disease has increased significantly over the last decade ***(1,2)***, and modern proteomic methods have evolved and been adapted to meet the demand. The specific challenges facing serum analysis include the wide dynamic range in the concentration of individual components and the tremendous number of potential variants of glycosylated proteins ***(3)***. The most dominant plasma proteins, albumin and immunoglobulin (Ig)G, typically comprise up to 70% of the plasma proteome in abundance. To enable the majority of the remaining, far less abundant proteins to be better visualized by two-dimensional gel electrophoresis (2-DE), these two proteins must first be removed or, at least, depleted in relative concentration. There are a number of currently available commercial products from a range of suppliers that enable albumin depletion by chemical affinity, exploiting the remarkable albumin-binding ability of structures closely related to the reactive dye molecule Cibacron blue 3GA ***(4)***, and the IgG binding properties of protein G ***(5)***. The blue dye has been shown to have a special affinity for proteins containing the dinucleotide fold, a structural feature that is common to several classes of proteins ***(4)***. Albumin can be separated from other plasma proteins using lectin affinity, as it is not normally glycosylated, while the majority of classical plasma proteins are. This approach allows both enrichment of lower-abundance proteins, and the study of differences in glycoprotein profiles ***(6)***. Highly effective depletion of albumin using monoclonal antibody selection has also been demonstrated, and coupled with protein G/IgG depletion ***(7)***.Recent research has identified 325 distinct proteins from 1800 2-D gel protein features following multi-component immunoaffinity extraction and further comprehensive chromatographic fractionation ***(8)***. Depletion of IgG by

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a protein G resin can also be coupled with NaCl/Ethanol precipitation to deplete albumin ***(9)***. However, depletion can cause problems in itself. The concentration of the high abundant proteins varies considerably within bodily fluids. Therefore once the samples are depleted, there is a significant change in the relative concentration of the protein constituents from the original sample. As an example in our own studies the percentage protein remaining after depletion in cerebrospinal fluid has an average of 17.9% with a standard deviation of 6.6%. This could be overcome by loading according to sample volumes rather than protein loading, which is often the norm in proteomic experiments ***(10)***. A major drawback is that the depletion not only adds further steps in sample processing, but undoubtedly alters the protein constituents within the sample as high abundant proteins bind other proteins. Consequently, the depletion may complicate quantitative comparisons and result in the loss of potential biomarkers ***(11)***.

2. Materials

1. Blue dye affinity resin: Affi-gel, (Biorad), Mimetic Blue SA (Prometic).
2. 20 m*M* Na_2HPO_4, pH adjusted to 7.0 with HCl.
3. Lysis buffer: 7 *M* urea, 2 *M* thiourea, 30 m*M* Tris, 5 m*M* magnesium acetate, 4% CHAPS, 1% NP-40.
4. Trichloroacetic acid (TCA)/acetone protein precipitation: 2-D Cleanup Kit (Amersham Biosciences, Piscataway, NJ).
5. Biomax 10K NMWL membrane centrifugal filter (Millipore, Bedford, MA).
6. WGA-agglutinin lectin (Sigma, St. Louis, MO).
7. Handee Mini Spin Columns (Pierce Biotechnology, Inc., Rockford, IL) or Macro Spin Columns (NEST Group, Southborough, MA).
8. Phosphate buffer: 50 m*M* Na_2HPO_4, 0.2 *M* NaCl, pH adjusted to 7.0 with HCl.
9. Phosphate buffer with SDS: 50 m*M* Na_2HPO_4, 0.2 *M* NaCl, 0.05% SDS, pH adjusted to 7.0 with HCl.
10. Sugar solution: 0.3 *M* *N*-acetyl glucosamine or neuraminic acid (Sigma, St. Louis, MO), in phosphate buffer.
11. Resin slurry: Albumin and IgG Removal Kit (Amersham Biosciences, Piscataway, NJ).
12. POROS Affinity depletion cartridges (Applied Biosystems, Framingham, MA).
13. AccuGENE 10X PBS solution (Cambrex, East Rutherford, NJ).

3. Methods

3.1. Affinity Depletion of Serum Albumin

3.1.1. Batch Use of Dye-Agarose Affinity Resins

Here we describe a generic method, based on manufacturer's instructions, adaptable for the majority of available dye resin slurries. The steps involved are equilibration, binding, washing, and stripping.

1. Add 250 µL of resin (*see* **Note 1**) to a 1.5-mL microcentrifuge tube (*see* **Note 2**), centrifuge for 2 min at 0.2 *g*, and remove liquid using a gel-loading pipet tip.
2. Add 200 µL of 20 m*M* sodium phosphate, pH 7.0, and shake gently for 10 min. Centrifuge at 1000 rpm for 2 min and remove liquid. Repeat the equilibration twice.
3. Take 25 µL of clarified plasma/serum, dilute it with 175 µL of the 20 m*M* sodium phosphate, pH 7.0 buffer, and mix thoroughly.
4. Add the diluted plasma to the conditioned resin, vortex briefly, and shake gently for 10 min to allow serum albumin to bind to the dye resin. Centrifuge for 2 min at 1000 rpm and remove supernatant using a gel-loading pipet tip. Transfer solution, containing unbound albumin-depleted proteins, to a clean 1.5-mL microcentrifuge tube.
5. Wash the resin three times with 150 µL 20 m*M* sodium phosphate, pH 7.0 buffer, shake for 10 min, centrifuge for 2 min at 1000 rpm, remove the supernatant, and combine with the previous solution.
6. Elute the proteins bound to the resin by washing three times with lysis buffer (*see* **Note 3**). Combine stripped fractions into a separate 1.5-mL tube.
7. Concentrate the albumin-depleted fraction (approx vol 650 µL) to a final vol of approx 100 µL using a 10,000 MWCO (molecular weight cut-off) membrane filter. Desalt and prepare for 2-DE by TCA/acetone precipitation (*see* **Note 4**). For the albumin-rich fraction, remove a 150-µL aliquot from the stock solution and perform TCA/acetone precipitation directly.

3.1.2. Lectin Affinity Serum Albumin Removal Using WGA/Agarose

1. Prepare the Handee Mini Spin column for use by ensuring the frit at the bottom of the main chamber is firmly in place by pushing down with a paper clip. Resuspend the lectin in the buffer supplied by the manufacturer to give a homogeneous lectin slurry, then pipet 200 µL into the chamber, centrifuge briefly, and remove liquid.
2. Add 500 µL of phosphate buffer and shake gently for 10 min. Centrifuge at 1000 rpm for 2 min, remove liquid, and repeat twice.
3. Prepare plasma by diluting 50 µL of plasma with 750 µL of phosphate buffer containing 0.05% SDS. This amount will typically yield 50 µg of albumin-free protein.
4. Transfer diluted plasma to the column containing the beads. Shake gently using a rotary mixer, ensuring good mixing, for 5 min. Centrifuge briefly and collect eluent. Wash beads three times with 200 µL of phosphate buffer. Combine wash eluents (albumin-rich fraction).
5. Elute the glycosylated proteins with 200 µL sugar solution *see* **Note 6**), shaking for 5 min on a rotary shaker. Wash three times with 150 µL of sugar solution and combine, total volume approx 650 µL.
6. Reduce the albumin-depleted fraction to a final vol of approx 100 µL using a 10,000 MWCO membrane filter. Desalt and prepare for 2-DE by TCA/acetone

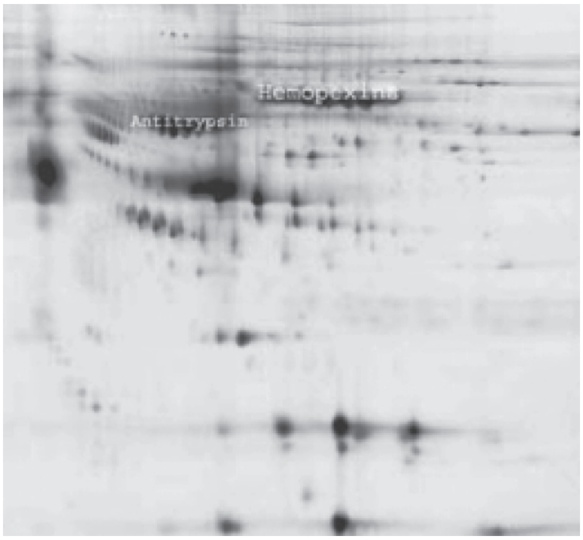

Fig. 1. An example of a two-dimensional gel showing serum prepared using the method outlined in **Subheading 3.1.2**.

precipitation (*see* **Note 4**). An example of a 2-D gel showing serum prepared by the method in **Subheading 3.1.2**. is shown in **Fig. 1**.

3.1.3 Antibody Serum Albumin/Immunoglobulin G Removal

3.1.3.1. Use of Antibody/Protein G Resin

1. Pipet 20 µL of plasma/serum into a 1.5-mL microcentrifuge tube and add 750 µL of resin slurry. Close cap and mix on a rotary shaker, ensuring strong mixing is taking place, for 30 min at room temperature.
2. Transfer entire slurry to the upper chamber of a microspin column and insert column into a suitable microcentrifuge tube. Centrifuge at 1000 rpm for 5 min. Remove liquid (approx 500 µL) and desalt/concentrate as described in **Subheading 3.1.1., step 7**.

3.1.3.2. Use of Antibody/Protein G Cartridges (see **Note 7***)*

1. Connect a syringe to the anti-SA cartridge using a needle-port adapter and equilibrate packing material with at least five cartridge volumes of PBS solution, pH 7.4.
2. Dilute the serum/plasma 10-fold with PBS solution and inject an amount less than the maximum binding capacity into the cartridge at a steady flow rate (*see* **Note 8**).
3. Collect the flowthrough in a clean 0.5-mL microcentrifuge tube. Wash cartridge with three column vols of PBS solution and combine wash fractions in a separate tube.

4. Connect the needle-port adapter to the protein G cartridge and condition the packing material with at least five cartridge vols of PBS solution, pH 7.4.
5. Inject the albumin-depleted fraction from **step 3** at a steady flow rate and collect the flowthrough in a fresh 1.5-mL tube.
6. Inject the combined wash fractions from **step 3** at a steady flow rate and combine with liquid from **step 6**.
7. Concentrate the albumin-IgG-depleted fraction to a final volume of approx 100 µL using a 10,000 MWCO membrane filter. Desalt and prepare for 2-DE by TCA/acetone precipitation (*see* **Note 4**).

3.2. Use of Commercial Depletion Kits

3.2.1 Commercial Kits Available

There are many different commercial depletion kits available which include Aurum Serum Protein Minikit (Bio-Rad, Hercules, CA), Multiple Affinity Removal Column (Agilent Technologies, San Diego, CA), POROS Affinity depletion Cartridges (Applied Biosystems, Framingham, MA) and Albumin and IgG Removal kit (Amersham Biosciences, Uppsala, Sweden). The relative performance of these columns has been compared by Bjorhall *et al.* ***(12)***.

3.2.2 Protocol Using the Agilent Multiaffinity Depletion Column

1. Add an appropriate volume of bodily fluid to a 4 ml spin column.
2. Centrifuge sample at 4000 rpm for 30 min..
3. Add 1 ml of buffer A (Agilent Technologies).
4. Centrifuge sample at 4000 rpm for 30 min.
5. Repeat **steps 3** and **4** a further two times.
6. Filter CSF solution through a 0.22 micron spin filter.
7. Centrifuge sample through the column at 100 g for 1.5 min and collect elute.
8. Add 400 mcl Buffer A and centrifuge through column at 100 g for 2.5 min and collect elute.
9. Repeat **step 8**.
10. Keep total elute (1 ml) which contains the depleted sample.
11. Elute bound fraction by passing 2 ml Buffer B, (Agilent Techonologies) slowly through column.
12. Buffer exchange samples into an appropriate buffer for downstream analysis. Representative 2-D gel images of cerebrospinal fluid, before and after depletion with the above described column, are shown in **Fig. 2**.

4. Notes

1. The amount of resin used here ensures that the total albumin loaded to the resin is less than its albumin-binding capacity (typically approx 11 mg/mL).
2. As an alternative to the procedure listed here, the resin may be applied to the upper chamber of a spin column, and the removal of liquid facilitated

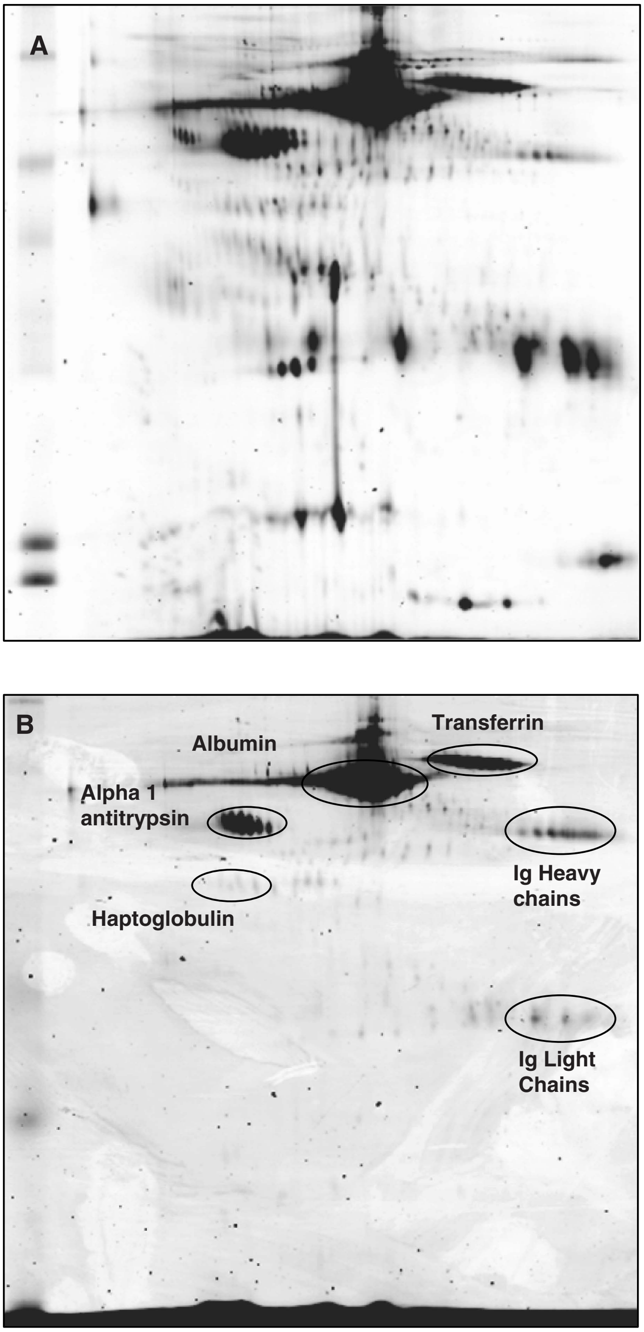
A
B
Albumin
Transferrin
Alpha 1 antitrypsin
Ig Heavy chains
Haptoglobulin
Ig Light Chains

Fig. 2. (continued)

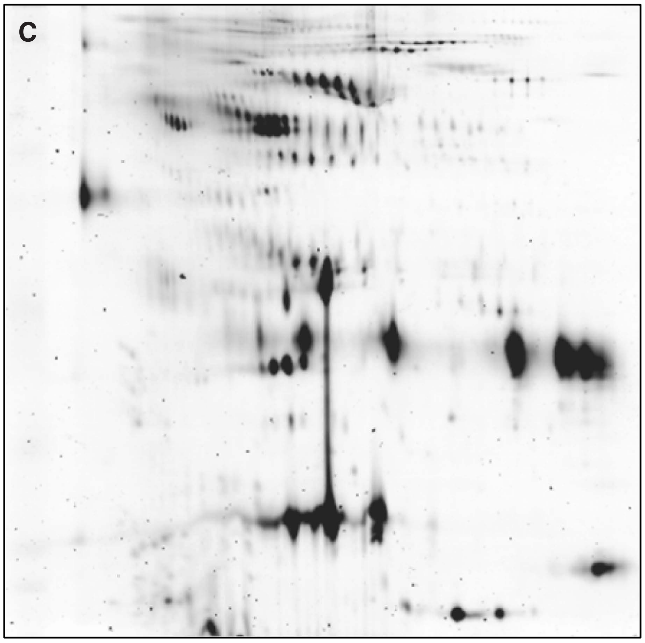

Fig. 2. 2-D gel images of cerebrospinal fluid before and after antibody depletion. Depletion was carried out using commerical depletion columns (Agilent Technologies). Representative images of the original sample **(A)**, the bound fraction **(B)**, and the depleted fraction **(C)**.

by centrifugation. In our hands, this variation led to less efficient albumin removal due to inconsistent residence times.

3. Alternatives to lysis buffer include solutions of sodium chloride or guandinium chloride (5–6*M*).
4. Desalting/concentrating is absolutely required for the isoelectric focusing step. The use of MWCO membrane filters followed by protein precipitation was found in our hands to be the most effective method combination. The initial preconcentration to volumes of 100μL depletes salt levels and reduces the amount of protein precipitation solutions required.
5. Columns are available in several sizes and therefore total protein loading capacities.
6. *N*-acetyl glucosamine can be replaced by *N*-acetyl neuraminic acid depending on the type of glycosylation under investigation.
7. These cartridges can be used separately as outlined here, or in series, either manually with the aid of a syringe, or automatically using a solvent delivery system on a liquid chromatograph.

8. The albumin-binding capacity depends on the column size and will be supplied by the manufacturer. The albumin content of the plasma can only be estimated, unless specific albumin assay methods are available.

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Immunoaffinity Depletion of High Abundance Plasma and Serum Proteins

Lynn A. Echan and David W. Speicher

1. Introduction

Analysis of human serum and plasma has been a major focus of many recent proteomics studies because blood is a promising, routinely collected biological fluid that is likely to contain multiple novel biomarkers for many human diseases, such as cancers and cardiovascular disease ***(1)***. Most cells in the body are thought to secrete or shed proteins into the blood, and therefore plasma is very likely to contain information concerning the physiological status of all tissues and organs in the body ***(2–4)***. While serum and plasma hold great potential as sources of new disease biomarkers, systematic discovery of novel biomarkers in patient cohorts is greatly complicated by the presence of a small number of highly abundant (mg/ml range) proteins in blood ***(2–6)***. In particular, serum albumin constitutes about 65% of total serum protein, while immunoglobulins contribute an additional 15%. Most of the remaining 20% of total protein content is comprised of a modest number of additional high- and medium-abundance (μg/ml range) proteins, which tend to obstruct discovery of novel, specific disease biomarkers present at ng/ml to pg/ml levels.

Until approximately 2003, the only available technology for depletion of major proteins was represented by commercial kits that combined Cibacron blue dye for removal of albumin with Protein A or G for removal of immunoglobulins. Unfortunately, these columns incompletely removed albumin and removed many non-targeted proteins, including potential biomarkers ***(7)***. Recently, several companies have produced immunoaffinity depletion resins that are more effective and more specific than the blue dye approach for depleting multiple blood proteins. Depletion of at least six or more abundant proteins has become standard practice as the first step in multidimensional

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plasma or serum proteome analyses. Early research studies explored use of monoclonal or polyclonal antibodies to attempt to remove one or multiple abundant proteins. The development *(8)* and commercial production of the Multiple Affinity Removal System (Agilent) in 2003 was particularly noteworthy as it was the first widely available product that removed more than one or two proteins. Specifically, it removed six abundant proteins and reduced the protein content by 85%. This improves protein profiling capacities substantially, but the next level of abundant proteins continue to limit volumes of plasma that can be analyzed. Hence, ideally additional abundant proteins should be depleted so that at least 98–99% of total protein is removed *(4,7)*.

More recently, several manufacturers have produced antibody columns against 7, 12, 14, or 20 abundant blood proteins in both HPLC/FPLC and spin column formats. In addition, most manufacturers will produce custom larger scale FPLC or spin column formats to fit the needs of individual laboratories. The choice of FPLC or spin column format depends upon several factors. First, FPLC columns require relatively expensive instrumentation not available in every laboratory, and antibody LC columns are usually larger and more expensive than spin columns. However, the FPLC columns are highly robust, more completely remove the targeted proteins, and are more time efficient because they usually deplete larger volumes of plasma or serum in a single pass.

In our experience, the available commercial multiprotein immunodepletion columns (**Table 1**) perform similarly. That is, they are highly efficient at depleting the targeted proteins, typically >98–99.5% removal of most targets, while simultaneously not removing significant amounts of untargeted minor proteins *(7)*. Of course, if serum albumin is present at 40 mg/ml and 99% is removed, the residual 0.4 mg/ml of albumin is still a major protein. Hence, for the most critical experiments, it is necessary to optimize removal to greater than 99%, at least for the most abundant proteins. In this regard, it is not surprising that polyclonal antibodies are usually used for these depletion matrices rather than monoclonal antibodies. Monoclonal antibodies are sometimes more specific; each antibody has a single binding affinity, and it is easier to consistently produce a uniform product. But because they have single epitopes, they will usually be less effective than polyclonal antibodies in removing proteolytic fragments, some posttranslationally modified forms of the target protein, oxidized forms of the protein, etc. An unknown challenge is whether these commercial products can be consistently produced over the long term, since polyclonal antibody properties can vary from batch-to-batch due to variations in host immune response to injected antigens.

Factors that may influence decisions concerning the type of column to use include: FPLC or spin format, column size and capacity options, moderate

Table 1
Commercially Available Multiprotein Immunoaffinity Depletion Columns[a]

Manufacturer/Product	# Proteins Depleted	Format	Capacity	# Runs[b]
Sigma				
ProteoPrep 20 (Human)	20	10 ml LC Spin Column	100 µl 8 µl	100
Agilent MARS Human-14	14	4.6 × 50 mm LC	20–40 µl	200
		4.6 × 100 mm LC	20–40 µl	
		10 × 100 mm LC	160 µl	
		Std. Spin Column	8–10 µl	
MARS Human-7	7	4.6 × 50 mm LC	30–70 µl	200
		4.6 × 100 mm LC	30–70 µl	
		10 × 100 mm LC	250–300 µl	
		Std. Spin Column	12–14 µl	
MARS Human-6	6	4.6 × 50 mm LC	15–80 µl	200
		4.6 × 100 mm LC	15–80 µl	
		10 × 100 mm LC	340 µl	
		Std. Spin Column	7–10 µl	
		HC[c] Spin Column	14–16 µl	
MARS Mouse-3	3	4.6 × 50 mm LC	37–100 µl	200
		4.6 × 100 mm LC	37–100 µl	
		Std. Spin Column	25–30 µl	
Beckman IgY- 12 (Human)	12	2 ml LC	50 µl	100
		10 ml LC	250 µl	
		Spin Column	20 µl	
IgY-R7 (Mouse/Rat)	7	2 ml LC	20–40 µl	100
		10 ml LC	100–200 µl	
		Spin Column	10–15 µl	

[a] Only products that deplete at least six abundant human proteins and the only available mouse columns are summarized here. Some columns only deplete one or two proteins.

[b] Stated maximum lifetime as reported by manufacturer.

[c] High capacity

price-to-capacity differences, whether buffers are defined or proprietary, and most importantly, the proportion of the total plasma protein removed by the column. As a general rule, it is beneficial to deplete as many abundant proteins as possible, because the greater the reduction in total protein content per volume of original plasma, the greater the improvement in detection of lower abundance proteins. The following protocols describe common HPLC/FPLC and spin column immunoaffinity depletion using ProteoPrep 20 columns, which currently remove the largest number of proteins and remove approximately 95% of the total plasma protein content. The same methods can be used for either plasma or serum. For simplicity, we will generally refer to plasma only when describing the protocols.

2. Materials

2.1. FPLC Affinity Depletion of Plasma or Serum

1. Human plasma or serum.
2. HPLC or FPLC system capable of operating at low pressure.
3. ProteoPrep 20 LC Plasma Immunodepletion Column Kit (Sigma Catalog No. PROT20LC). Storage: 2–8 °C. Individual components are:
    a. ProteoPrep 20 LC Plasma Immunodepletion Column; 9.4 ml resin, 100 µl plasma (50–70 mg) capacity (Sigma Catalog No. P9874).
    b. ProteoPrep 20 Equilibration Buffer, 10x concentrate; (Sigma Catalog No. P1749).
    c. ProteoPrep 20 Elution Solution, 10x concentrate; (Sigma Catalog No. P9749).
    d. ProteoPrep Preservative Concentrate; (Sigma Catalog No. K3889).
4. Stericup GP Express Plus Membrane, 0.22 µm filter units (500 ml capacity); (Millipore Catalog No. SCGPU05RE).
5. Amicon Ultrafree-MC 0.22 µm microcentrifuge filters; (Millipore Catalog No. UFC30GVNB).
6. Milli-Q water or equivalent.

2.2. Spin Column Affinity Depletion of Plasma or Serum

1. Human plasma or serum.
2. ProteoPrep 20 Plasma Immunodepletion Kit (3 column kit: Sigma Catalog No. PROT20; Single column kit: Sigma Catalog No. PROT20S); Storage: 2–8 °C, Individual components are:
    a. ProteoPrep 20 Plasma Immunodepletion Spin Column (300 µl resin, 8 µl plasma capacity).
    b. ProteoPrep 20 Equilibration Buffer, 10× concentrate (Sigma Catalog No. P1749).
    c. ProteoPrep 20 Elution Solution, 10× concentrate (Sigma Catalog No. P9749).
    d. ProteoPrep Preservative Concentrate (Sigma Catalog No. K3889).
3. Luer Lock caps.
4. Several 5 or 10 ml syringes.
5. Amicon Ultrafree-MC 0.22 µm microcentrifuge filters (Millipore Catalog No. UFC30GVNB).
6. Amicon Ultra-4, 5 kDa MWCO centrifugal filter device (15 ml capacity) (Millipore Catalog No.UFC800524).
7. Milli-Q water or equivalent.

2.3. Large-Scale Spin Column Affinity Depletion of Plasma or Serum

1. Human plasma or serum.
2. ProteoPrep 20 Custom Plasma Immunodepletion Kit; Storage: 2–8 °C, Individual components are:
    a. ProteoPrep 20 Jumbo Plasma Immunodepletion Spin Column (3.7 µl resin, 100 µl plasma capacity).
    b. ProteoPrep 20 Equilibration Buffer, 10x concentrate (Sigma Catalog No. P1749).
    c. ProteoPrep 20 Elution Solution, 10x concentrate (Sigma Catalog No. P9749).
    d. ProteoPrep Preservative Concentrate (Sigma Catalog No. K3889).
3. Luer Lock caps.
4. Several 20 ml syringes.
5. Amicon Ultrafree-CL 0.22 µm microcentrifuge filters, 2 ml capacity (Millipore Catalog No.UFC40GV00).
6. Amicon Ultra-4, 5 kDa MWCO centrifugal filter device (Millipore Catalog No.UFC800524).
7. Milli-Q water or equivalent.

2.4. Ethanol Precipitation

1. Unbound or bound fraction from Prot-20LC or spin column depletion.
2. 200 proof Ethanol, chilled to −20°C (Sigma).
3. Speed-vac centrifuge.

3. Methods

3.1. FPLC Affinity Depletion of Plasma or Serum

Many HPLC systems require pressures greater than 100 psi to operate properly. The ProteoPrep 20 LC column packing has a rated pressure maximum of 30 psi. Hence, an FPLC-type system, capable of operating at low pressures and where a high pressure limit of 30 psi can be accurately set, is strongly preferred. If the available instrument requires higher minimum operating pressures, one option is to generate necessary backpressure in front of the column with a flow restrictor such as an appropriate length of small internal diameter PEEK tubing (e.g. 1–2 feet, 0.005 or 0.007 inch i.d.) and larger ID PEEK tubing (0.030 or 0.040 inch i.d.) after the column to avoid exposing the column to high pressures. Care must be taken to set the maximum pressure limit only slightly above the operating pressure to reduce the risk of compressing the column bed. Fortunately, the column itself has a much higher pressure rating than the column matrix. Hence, if an obstruction in the column or downstream does occur, there is minimal risk of a column explosion as long as the maximum pressure is set well below the pressure limit of the column housing.

1. Prepare Buffer A (1x Equilibration Buffer): Dilute one part of ProteoPrep 20 Equilibration Buffer, 10x concentrate with nine parts of room temperature ultrapure water. Filter the diluted buffer through a 0.22 µm filter unit and degas for 5 min. at room temperature using vacuum filtration with stirring. The final volume needed for each depletion is approximately 55 ml.
2. Prepare Buffer B (1x Elution Solution): Dilute one part of ProteoPrep 20 Elution Solution, 10x concentrate with nine parts of room temperature ultrapure water. Filter the diluted buffer through a 0.22 µm filter and degas for 5 min. at room temperature using vacuum filtration with stirring. The final volume needed for each depletion is approximately 30 ml.
3. Before connecting the column, flush Buffer A through the system to the column inlet line for 15 min at 1 to 3 ml/min. Transfer column to room temperature at this time.
4. Connecting column: to prevent air from being introduced into the column, after flushing the system as described in **step 3,** stop flow, remove the plug from the top of the column only, start the pump at 0.5 ml/min, and while holding the column inlet tubing over the top end of the column, fill the column end fitting with Equilibration Buffer. Stop flow and connect tubing to column inlet.
5. Remove the screw plug from the bottom of the column, connect the detector inlet solvent line to the column bottom, and equilibrate with Buffer A for 5 min at 1 ml/min (*see* **Note 1**).
6. Filter undiluted plasma (generally 100 µl) with a prerinsed 0.22 µm microcentrifuge filter (*see* **Note 2**). Keep filtered sample on ice until ready for use (*see* **Note 3**).
7. Prior to injection of the first sample of the day, run a blank run (no injection) and reequilibrate the column using Buffer A (*see* **Note 4**).
8. Inject 100 µl of filtered plasma at 0.3 ml/min.
9. Elute the depleted plasma (unbound fraction) from the column with Buffer A at 0.3 ml/min. Collect the flow-through fractions and pool the unbound fractions containing protein as it comes off the column (*see* **Note 5**). Keep the pooled sample on ice.
10. Elute bound proteins with Buffer B at 1 ml/min. Collect and pool bound proteins and store on ice (*see* **Note 6**).
11. Reequilibrate column with Buffer A for 10 min at 3 ml/min.
12. Inject the next sample and repeat **steps 8 to 11** until all samples are separated or a sufficient volume of a single sample is depleted.
13. Disconnect and store the column at 4°C in Buffer A. (*see* **Note 7**).
14. Replace Buffers A and B with ultrapure water and flush system, including detector, for 30 min at 1 ml/min followed by 50% isopropanol for 30 min at 1 ml/min (*see* **Note 8**).

3.2. Spin Column Affinity Depletion of Plasma or Serum

There are several strategies for spin-column usage. First, to maximize column load, it is possible to moderately overload the column, which results in depletion of about 90–95% of the targeted proteins. The unbound fraction is then repurified on either the same column or a duplicate column to “polish” the purification. The repurification step can be conducted on a single unbound fraction after it has been concentrated, or multiple unbound fractions can be pooled, concentrated and repurified in a single repurification run. This two-pass method has the advantage of being able to deplete larger final volumes of a single sample per day. An alternative strategy is to load the spin column at or slightly below the recommended capacity and conduct one or more single-pass purifications. If the plasma concentration is not too high (column capacities are rated by vendors based on volume, not protein concentration), the final sample or pool of multiple samples may not need to be redepleted. However, they will usually need to be concentrated and one is likely to only achieve 95% depletion of targeted proteins using this method. A third and most conservative strategy is to both underload the column and redeplete the initial unbound fraction in a final polishing step. This should provide at least 99% removal of most targeted proteins, but volume of plasma purified per day will be somewhat reduced.

The method presented here is the manufacturer's recommended approach to maximize volume of plasma depleted per given time period. Multiple aliquots of a plasma sample (up to 10) are depleted, pooled, and concentrated, and following elution of the bound proteins, the unbound fraction from all 10 samples is redepleted in a polishing step. While not all vendors recommend a polishing step, the principle of removing small amounts of residual targeted proteins in the unbound pool using a repurification is generally applicable and is a good idea for the most critical work, especially when spin columns are used.

1. Prepare Buffer A (1x Equilibration Buffer): Dilute one part of ProteoPrep 20 Equilibration Buffer, 10x concentrate with nine parts of room temperature ultrapure water. The final volume necessary for each plasma application is 5 ml.
2. Prepare Buffer B (1x Elution Solution): Dilute one part of ProteoPrep 20 Elution Solution, 10x concentrate with nine parts of room temperature ultrapure water. The final volume necessary for each plasma application is 2 ml.
3. Remove the bottom plug and screw cap from the spin column and place into a 2 ml collection tube. Centrifuge the spin column at 1000–2000 × g for 60 sec to remove the storage buffer using either a speed-vac without vacuum or a microfuge.
4. Attach a Luer Lock fitting to the spin column, draw 4 ml of 1x Equilibration Buffer into a syringe, attach the syringe to the Luer Lock cap, and slowly push the buffer through the column to equilibrate the resin.
5. Remove the Luer Lock fitting, loosely recap the column, and place into a collection tube. Centrifuge the spin column at 1000–2000 × g for 60 sec to remove the excess Equilibration Buffer. Discard the buffer and place the column into a clean collection tube.
6. Dilute plasma sample (generally 8 µl per depletion) to 100 µl with Equilibration Buffer (*see* **Note 9**). Filter diluted plasma with a prerinsed 0.22 µm microcentrifuge filter. Keep filtered sample on ice until ready for use.
7. Add 100 µl diluted sample to the top of the packed resin bed, and incubate at room temperature for 20 min.

8. Centrifuge the spin column and collection tube at 1000–2000 × g for 60 sec.
9. Wash the remaining depleted plasma from the column with 100 µl 1x Equilibration Buffer. Collect wash by centrifuging the spin column at 1000–2000 × g for 60 sec. This wash may be collected in the same tube used in **step 8.**
10. Repeat **step 9** for an additional 100 µl wash of the resin, the final pooled volume of the unbound fraction should be approximately 300 µl.
11. Elute bound proteins with 1x Elution Solution. Attach a Luer Lock fitting to the spin column, draw 2 ml of 1x Elution Solution into a syringe, and slowly push the buffer through the column to remove proteins bound to the resin.
12. Neutralize bound proteins with 1M NaOH to bring pH to 8.0 (*see* **Note 6**).
13. Reequilibrate the spin column: Attach a Luer Lock fitting to the spin column, draw 4 ml of 1x Equilibration Buffer into a syringe, and slowly push the buffer through the column to equilibrate the resin.
14. Remove the Luer Lock fitting and place the column into a clean collection tube. Centrifuge the spin column at 1000–2000 × g for 60 sec to remove the excess Equilibration Buffer. Discard the buffer and place the column into a new collection tube.
15. Repeat **steps 7–14** for up to 10 depletions.
16. After all samples have been depleted, begin concentrating the unbound fraction (*see* **Note 10**). Add up to 4 ml of unbound pool to a prerinsed Amicon-Ultra 5K MWCO concentrator. Centrifuge for 15 min at 1500 × g, 4°C.
17. Repeat **step 16** until the unbound pool is concentrated to 100–200 µl.
18. Rinse the concentrator unit with 100 µl Equilibration Buffer, and pool this with the concentrated unbound sample to recover proteins adhering to the concentrator membrane.
19. Add 100 µl of the unbound sample to the reequilibrated and packed resin bed. Incubate for 20 min and centrifuge spin column and collection tube at 1000–2000 × g for 60 sec.
20. Repeat **step 19** until all of the concentrated unbound plasma has been reapplied to the column.
21. Wash the column twice with 100 µl Equilibration Buffer, centrifuge, and pool washes with the twice-depleted plasma. The final volume should be about 500 µl.
22. Repeat **steps 11–14** from this section to elute remaining bound proteins and reequilibrate the column.
23. Store column in 300 µl Equilibration Buffer containing Preservative Solution, with the bottom plug securely in place, and tightly recap the column (*see* **Note 7**).

3.3. Large-Scale ProteoPrep 20 Spin Column Depletion Using a Jumbo Custom Column

As mentioned above, some manufacturers will create custom HPLC or spin columns to meet the needs of investigators who want to deplete larger volumes of plasma or serum more efficiently. This protocol utilizes a custom ProteoPrep 20 Jumbo Plasma Immunodepletion Column (3.7 ml resin). Similar to the smaller spin column, there are several alternative loading/depletion strategies. For example, this column can be used to deplete 100 µl human plasma without a polishing step, and in one day it is practical to perform approximately 10 first pass depletions for a total volume of 1 ml per day. Alternatively, one can purify 250 µl per cycle and four first pass depletions followed by one polishing cycle of the combined and concentrated unbound fractions (also 1 ml but only 5 total cycles rather than 10 using the single-pass strategy).

1. Prepare Buffer A (1x Equilibration Buffer): Dilute one part of ProteoPrep 20 Equilibration Buffer, 10x concentrate with nine parts of room temperature ultrapure water. The final volume necessary for each plasma application is 60 ml.
2. Prepare Buffer B (1x Elution Solution): Dilute one part of ProteoPrep 20 Elution Solution, 10x concentrate with nine parts of room temperature ultrapure water. The final volume necessary for each plasma application is 24 ml.
3. Remove the bottom plug and screw cap from the spin column and place into a collection tube. Centrifuge the spin column at 1000–2000 × g for 60 sec to remove the storage buffer.
4. Attach a Luer Lock fitting to the spin column, draw 16 ml of 1x Equilibration Buffer into a syringe, attach the syringe to the Luer Lock cap, and slowly push the buffer through the column to equilibrate the resin.
5. Remove the Luer Lock fitting, loosely recap the column, and place into a collection tube. Centrifuge the spin column at 1000–2000 × g for 60 sec to remove the excess Equilibration Buffer. Discard the buffer and place the column into a clean collection tube.
6. Dilute plasma sample (100 or 250 µl per depletion) to 1.25 ml with Equilibration Buffer. Filter diluted plasma with a prerinsed 0.22 µm centrifuge filter. Keep filtered sample on ice until ready for use.
7. Add 1.25 ml diluted sample to the top of the packed resin bed, and incubate at room temperature for 20 min
8. Centrifuge the spin column and collection tube at 1000–2000 × g for 60 sec.
9. Wash the remaining depleted plasma from the column with 1.25 ml 1x Equilibration Buffer. Collect wash by centrifuging the spin column at 1000–2000 × g for 60 sec. This wash may be collected in the same tube used in **step 8.**
10. Repeat **step 9** for an additional 1.25 ml wash of the resin, the final pooled volume should be about 3.75 ml.
11. Elute bound proteins with 1x Elution Solution. Attach a Luer Lock fitting to the spin column, draw 12 ml of 1x Elution Solution into a syringe, and slowly push the buffer through the column to remove proteins bound to the resin.
12. Centrifuge the spin column in collection tube at 1000–2000 × g for 60 sec to remove any additional Elution Solution from the column.
13. Neutralize bound proteins with 1M NaOH to bring pH to 8.0 (*see* **Note 6**).
14. Reequilibrate the spin column: Attach a Luer Lock fitting to the spin column, draw 16 ml of 1x Equilibration Buffer into a syringe, and slowly push the buffer through the column to equilibrate the resin.

15. Remove the Luer Lock fitting and place the column into a clean collection tube. Centrifuge the spin column at 1000–2000 × g for 60 sec to remove the excess Equilibration Buffer. Discard the buffer and place the column into a new collection tube.
16. Repeat **steps 7–14** for up to 10 depletions of 100 µl or 4 depletions of 250 µl. Unbound fractions can be concentrated while subsequent depletions are being performed.
17. After all samples have been depleted, complete concentration of the unbound pool (*see* **Note 10**). Add up to 4 ml of unbound to a prerinsed Amicon-Ultra 5K MWCO concentrator. Centrifuge for 15 min at 1500 × g, 4°C.
18. Repeat **step 17** until the unbound sample is concentrated to approximately 1 ml.
19. Rinse the concentrator unit with 250 µl Equilibration Buffer, and pool this with the concentrated unbound sample to recover any proteins adhering to the concentrator membrane.
20. If 250 µl aliquots were purified per cycle, or if maximum depletion of 100 ml per cycle purifications are desired, add 1.25 ml of the unbound sample to the reequilibrated and packed resin bed. Incubate for 20 min and centrifuge spin column and collection tube at 1000–2000 × g for 60 sec.
21. Repeat **step 20** until all of the concentrated unbound plasma has been reapplied to the column.
22. Wash the column twice with 1.25 ml Equilibration Buffer, centrifuge, and pool washes with the twice-depleted plasma. The final volume should be 3.75 ml.
23. Repeat **steps 11–14** from this section to elute remaining bound proteins and reequilibrate the column.
24. Store column in 1.25 µl Equilibration Buffer containing Preservative Solution, with the bottom plug securely in place, and tightly recap the column (*see* **Note 7**).

3.4. Ethanol Precipitation

We have found that ethanol precipitation is an effective alternative to ultrafiltration for final unbound and bound fractions from either FPLC or spin columns that will be used in subsequent steps that use denaturants such as either 8 M urea or SDS. The ethanol precipitation requires less operator time and more effectively removes salts and detergent. The ethanol should be high quality, absolute proof, and must be chilled to −20°C to facilitate protein precipitation.

1. Add 9 volumes of 200 proof ethanol (−20°C) to the pooled unbound fraction.
2. Incubate overnight at 0°C.
3. Centrifuge sample for 30 min at 1500 × g, 4°C.
4. Carefully remove supernatant and air dry pellet briefly in a fume hood or speed vac (*see* **Note 11**).

4. Notes

1. The ProteoPrep LC column comes packed in a storage solution containing glycerol. For the first use of the column, attach the top of the column to the tubing as described in **Subheading 3.1, step 4.** Do not connect the

column outlet line to the bottom of the column. Instead, place the column over a beaker to collect the glycerol solution. Allow the column to equilibrate with 20–25 ml of 1x Equilibration Buffer at no higher than 0.5 ml/min. Carefully monitor pressure and decrease the flow rate as needed to not exceed 30 psi. Increased pressures at this step may cause bed compression and a void at the top of the resin bed. When the resin has changed from opaque to white, and the buffer runs clear, the column outlet line may be connected. The column should be conditioned with two complete blank cycles prior to injecting a sample. It is also preferable to run a few non-critical control plasma samples before depleting plasma samples that will be used in comparative studies.

2. Earlier versions of this method recommended diluting the plasma sample 5x with Buffer A prior to filtering. To enhance depletion of apolipoproteins and recovery of unbound proteins, the manufacturer recommended omitting the dilution step. However, we have not observed any major changes in apolipoprotein removal nor does a five-fold dilution of the plasma significantly increase the final volume of the unbound pool. Additionally, diluting the sample makes it more convenient to add protease inhibitors to the sample before injection if desired.
3. The manufacturer describes this column's capacity as 100 μl of plasma with a concentration of 40–50mg/ml. However, this is a very low estimate for protein concentration, and most human serum and plasma samples are likely to have substantially higher protein concentrations. For example, the range for total blood protein in normal human serum is 60–85 mg/ml, and plasma samples will have similar or slightly higher values. Furthermore, as indicated above, the normal range is quite broad and ranges in patients with diseases are often even more variable. Total protein concentration will also be affected by the use of different anti-coagulants and collection tubes. Therefore, it is advisable to determine the protein concentration of each sample prior to depletion and base loads upon total protein, not volume. When preparing samples, other considerations to note are: the number of depletions and/or different plasma samples that will be run in a single day. If multiple runs of the same sample are being depleted, it is best to thaw and prepare the entire sample at once and store it on ice until the entire sample is depleted, for optimal reproducibility. If several samples from different donors are being depleted sequentially, it is recommended that a single sample is thawed and filtered shortly before its injection, and while one sample is running, thaw and prepare the next sample. This will minimize proteolysis of the sample. A related option is to add protease inhibitors to the plasma sample prior to injection, although the value of adding protease inhibitors is ambiguous. Plasma and serum intrinsically contain many proteases as well as protease inhibitors, and some proteolysis

of susceptible proteins usually occurs in vivo. While further proteolysis may occur during sample processing, some protease inhibitors may react indiscriminately with plasma proteins ***(9)*** and other inhibitors are proteins or large peptides. Instead of adding protease inhibitors to plasma or serum, we prefer to minimize exposure of samples to conditions that favor proteolysis. The one step where it is not practical to avoid protease favorable conditions is during binding of the targeted proteins to the immunoaffinity resin. This is because in order to optimize binding affinity, conditions close to physiological pH without denaturants must be used, and in order to achieve reasonable binding kinetics, low temperatures should be avoided. However, it should be noted that plasma and serum are far more stable to proteolysis than cell lysates, and moderate length exposure to physiological conditions at room temperature appear to have only very minor effects on proteomes **(9).** Regardless of the precise sample handling methods to be used, the most critical factor is to ensure that all samples to be compared are processed in as consistent a manner as possible.

4. We have found that running an initial “quick blank gradient” at 3 ml/min for the entire gradient with no injection is useful for several reasons. First, it will ensure that the column is equilibrated to room temperature prior to the sample being injected. Also, as the column warms up, solvent outgassing may occur; but the increased flow rate forces any small air bubbles out of the column before large pockets can form and disrupt the column packing. Finally, a blank run at the beginning of the day removes any residual protein that may be bound to the column from previous depletions. Any protein peaks observed during the blank run should be run on 1-D SDS-PAGE and analyzed with silver stain.
5. It is useful to reserve a small aliquot of each fraction before pooling the unbound to analyze individual fractions on 1-D SDS-PAGE.
6. It is recommended that bound proteins typically be collected and frozen for potential future analysis. Prior to storage, the bound pool should be neutralized by adding 1M NaOH to bring the pH to approximately 8.0.
7. Proper storage and monitoring of antibody columns is essential for maintaining optimal column performance. Storage in Equilibration Buffer at 4°C is suitable for short-term storage, i.e., if the column will be reused within a few days. Long-term column storage (1 week or more) requires the addition of a Preservative Solution, included with the ProteoPrep 20-LC and spin columns. Alternatively, flushing the column with Buffer A containing 0.01% sodium azide should protect the antibody column from microbial growth. It is important to keep track of the total number of cycles run on each column. Periodically and especially when the upper limit of recommended uses has been reached, column performance should be critically evaluated. We have found the lifetime of well managed columns is

usually greater than the recommended lifetimes. In addition, as column capacity slowly and inevitably declines, it is usually adequate to simply reduce protein loads slightly to achieve the same degree of depletion as was obtained with a new column. To monitor antibody loss from the column, bound fractions from sample purifications and bound fractions from blank cycles after extended column storage should be periodically monitored by digesting an aliqout of the bound pool with trypsin followed by LC-MS/MS analysis. The resulting data should ideally be searched against an all species database. Alternatively, a human sequence database supplemented with all immunoglobulin sequences from the species of origin for the antibodies on the column should be used. In this manner, one can detect antibody bleed and/or proteolysis of the coupled antibodies.

8. After each use, the FPLC or HPLC system should be thoroughly flushed with water, followed by 50% Isopropanol. This will prevent corrosion due

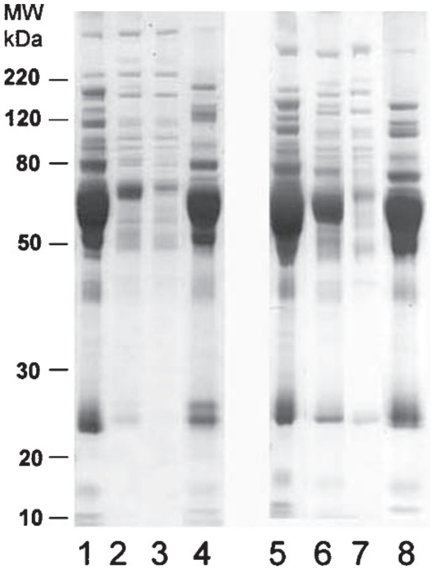

Fig. 1. Depletion of human plasma using spin columns. (***Left***) Results from depletion of ten 8 μl aliquots of human plasma using a standard spin column. Lanes: 1-undepleted plasma; 2-pool of depleted plasma from the ten runs after first cycle of depletion; 3-depleted plasma pool after polishing step; 4-pool of bound proteins eluted from column and concentrated using an Amicon-ultra 5K MWCO centrifugal filters. (***Right***) Results from depletion of a single 250 μl aliquot of human plasma using a large custom spin column. Lanes: 5-undepleted plasma; 6-unbound pool after first cycle of depletion; 7-unbound pool after polishing step; 8-pool of bound proteins after elution and concentration using an Amicon-ultra 5K MWCO centrifugal filters.

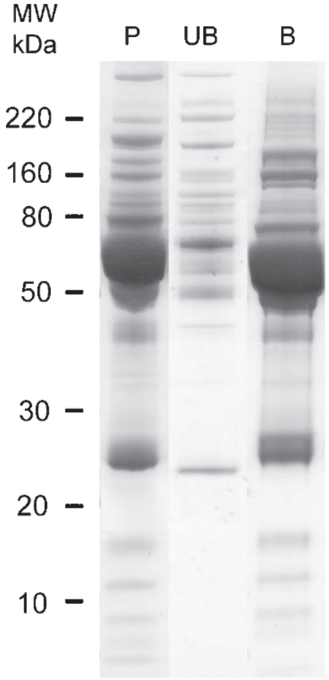

Fig. 2. Depletion of human plasma on a large LC column. Results from depletion of 100 μl human plasma after a single pass through a ProteoPrep 20 LC column. P-undiluted human plasma, UB-pooled unbound fraction after ethanol precipitation, B-pooled and neutralized bound fraction after ethanol precipitation.

to halogen salt-containing buffers or clogging of lines due to bacterial growth.

9. The spin column capacity is based on 8 μl of plasma with a concentration of 40–50mg/ml. If plasma concentration is higher, sample volume should be adjusted accordingly. If multiple aliquots of the same sample are to be depleted in one day, prepare enough sample for all depletions and store sample on ice. If proteolysis is a concern for downstream applications, it is acceptable to add protease inhibitors at this step (**Fig. 1**) (*see* **Note 3**).
10. Following each depletion, the manufacturer recommends continuous concentration of the unbound in the same micro-concentrator unit until the desired number of depletions has been completed. We prefer to pool the unbound, and begin concentration after all the depletions are completed, using a larger capacity concentrator. This will provide a total unbound sample before concentration that can be analyzed in parallel on 1-D

SDS-PAGE with the concentrated sample to check for protein losses during concentration.

11. To completely dry pellet, without risk of airborne contaminants or sample oxidation, it is preferable to dry the pellet under a gentle stream of argon or in a speed vac for a few minutes. The pellet is then resuspended in the desired resolubilization buffer, or the dried pellet may be stored at −20–80°C until it is needed. To check for potential sample loss during the ethanol precipitation, dry 10% of the ethanol supernatant in a speed vac, and run in parallel with proportional volumes of sample before and after precipitation on 1-D SDS-PAGE.

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Preparation of Yeast Samples for 2-D PAGE

Joakim Norbeck

1. Introduction

Yeasts are the focus of much research, both in their role as pathogens and as biotechnically important organisms, and not the least in their role as model systems for eukaryotic cells. In particular, *Saccharomyces cerevisiae* has also been the object of several proteomics-related efforts. However, the preparation of protein extract from yeast is complicated by the presence of a cell wall of mainly chitin and glucans, which needs to be disrupted in the extraction process. Several methods presenting solutions to this problem, in connection with sample preparation for two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), have been described ***(1,2)***, in which the cell wall is broken either by vortexing in the presence of glass beads or by sonication.

We have found the method described below to be robust and to yield reproducible results in several studies ***(3–6)***. It is furthermore easy to perform and does not require any specialized equipment.

2. Materials

1. Sample buffer I: sodium dodecyl sulfate (SDS) (0.3 g), β-mercaptoethanol (5.0 mL), Tris-HCl (0.444 g), Tris base (0.266 g), MilliQ (or equivalent) water (to a final volume of 10 mL).
2. Sample buffer II (*see* **Note 1**): 1.5 *M* Tris base (80 μL), 1.5 *M* Tris-HCl (1585 μL), 1 *M* $MgCl_2$ (250 μL), DNase I (Worthington Biochemical Corp., NJ) (5 mg), RNase A (Worthington Biochemical Corp., NJ) (1.25 mg), MilliQ (or equivalent) water (to a final volume of 5 mL).

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3. Immobilized pH gradient (IPG)-rehydration buffer (urea/thiourea buffer): urea (4.8 g; gives a final concentration of 8 *M*; *see* **Note 2**), Triton X-100 or Nonidet P40 (100 µL; *see* **Note 3**), 1 *M* DTT (100 µL), IPG-buffer/ampholine (50 µL; *see* **Note 4**), bromophenol blue (trace amount approx 0.01% w/v), MilliQ (or equivalent) water (to a final volume of 10 mL).

3. Method

The method described in the following sections can be divided into (1) an initial cell disruption step; (2) a protein solubilization step; (3) a nuclease treatment step; and (4) a final phase in which protein extract is diluted in IPG-rehydration buffer immediately prior to application on the first dimension of 2-D PAGE.

The procedure is carried out in 1.5-mL microcentrifuge tubes. A suitable starting material is a pellet of yeast cells from 10 mL of culture with a density of 5–10 million cells/mL, corresponding to an optical density (at 610 nm) of approx 0.5, which will typically yield a pellet of 5–10 µL of cells. The method described below is adjusted to this amount of cells.

The protocol can be scaled up or down; however, care should be taken to not use a final extract volume of more than 500 µL or less than 50 µL, since this will reduce the efficiency of the cell-disruption step.

3.1. Cell Disruption

1. Add 160 µL of ice-cold milliQ-quality water containing protease inhibitors (e.g., Complete™, Roche, Inc.) to the cell pellet.
2. Add 0.25 g of chilled glass beads (diameter 0.5 mm) to the sample.
3. Vortex 4 • 30 s on a table shaker at maximum speed (approx 2500 rpm) with intermittent placement of samples on ice for at least 1 min.

3.2. Protein Solubilization

1. Add 20 µL of sample buffer I and vortex the tube(s) briefly to mix.
2. Place tube(s) at 95°C for 5 min. Make sure to secure the lid of the tube, alternatively to make a small hole in the lid, prior to the heating step.
3. Cool samples on ice for 5 min.

3.3. Nuclease Treatment

1. Add 20 µL of sample buffer II and vortex the tube(s) briefly to mix.
2. Incubate on ice for 10 min.
3. Centrifuge samples at full speed (approx 15,000*g*) in a microcentrifuge at 4°C.
4. Aspire the supernatant (constituting the protein extract) to a new microcentrifuge tube and freeze at –20°C, or use immediately.

3.4. Dilution in IPG-Rehydration Buffer

1. Dissolve sample in required volume of IPG-rehydration buffer (*see* **Note 5**).
2. Incubate the sample at 37°C for 10 min.

3. Spin down sample (15,000*g*, 10 min, room temperature) to remove any particles that might remain.

4. Notes

1. The DNase and RNase should be dissolved in the buffer as the final step.
2. 8 *M* Urea can be substituted by a combination of 7 *M* urea and 2 *M* thiourea. The chaotropic agent thiourea is the most highly beneficial addition to the IPG-rehydration buffer; this addition can strongly improve the solubility of many proteins which may produce "streaking" or which are completely absent on 2-D PAGE gels run with normal urea-based buffer in the first dimension ***(7)***. However, thiourea requires a special permit from inspecting authorities in many countries due to its suspected carcinogenic properties.
3. Triton X-100 can be substituted for 2% (w/v) of CHAPS. CHAPS is considered to be the preferred detergent for the IPG-rehydration buffer ***(7)***. Unfortunately, it is also considerably more expensive than Triton X-100 and Nonidet P-40. We normally use Triton X-100, since in our hands the choice of detergent in the rehydration buffer has only a minor influence on the final protein resolution.
4. IPG buffer or ampholine should be chosen for each type of IPG strip, or for the desired separation interval.
5. The final volume to which the sample is diluted is determined by the system used for running this first dimension. We most often use the precast pH-gradient strips (supplied by, for example, Amersham Biosciences and BioRad, Inc.), for which the rehydration volume is commonly in the range of 125–500 µL. However, the protein extract is also compatible with the original glass tube-based system for running 2-D PAGE ***(8)***, or for application on IPG strips using sample cups, in which the sample volume will typically be <100 µL. In the simplest case, the desired amount of protein extract to be loaded is less than 10% of the final volume and contains low amounts of salt. The extract can then be mixed directly with IPG-rehydration buffer to the required volume immediately prior to application on the first dimension. If the desired amount of protein extract is greater than 10% of the final volume, or if the sample contains large amounts of substances that may disturb the isoelectric focusing (e.g., salts), additional steps such as precipitation or dialysis are required: A simple and straightforward way to concentrate the sample and remove low-molecular-weight compounds is the use of precipitation, either by acetone or trichloroacetic acid, or a combination of the two ***(9)***. The precipitate is then dissolved in a proper amount of IPG-rehydration buffer. Alternatively, the protein extract can be adjusted to a final concentration of 8 *M* urea (or 7 *M* urea/2 *M* thiourea), followed by dialysis against a large volume of IPG-rehydration buffer. In the latter case, a micro-dialysis kit with a cutoff of 1 kDa should be used (e.g., PlusOne™ Micro Dialysis kit,

Amersham Biosciences). Following dialysis, the sample is adjusted to the required volume, using IPG-rehydration buffer. However, it should be cautioned that both precipitation and dialysis may cause the loss of certain proteins from the extract. The amount of extract to be diluted also depends on whether a constant amount of protein (in µg) or a constant amount of radioactivity (in dpm of radioactively labeled amino acid) is desired. When measuring protein concentration in the extract, it is important to use a method that tolerates the presence of detergents and mercaptoethanol (e.g., a method which incorporates an initial protein precipitation step).

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18

Membrane Protein Preparation Using Aqueous Polymer Two-Phase Systems

Jens Schindler and Hans Gerd Nothwang

1. Introduction

Membrane proteins serve many fundamental functions for cells such as cell-cell interactions, signal transduction, and molecular transport both within and between cells. Bioinformatic analysis has estimated that transmembrane domain containing proteins represent about 20-30% of all open reading frames in sequenced genomes ***(1)***. In addition, many proteins are anchored to the membranes via lipidic posttranslational modifications such as glycosylphosphatidyl inositol anchors or via interaction with other membrane proteins. In total, embrane anchored proteins represent a considerable portion of all cellular proteins. They also represent 70% of all known drug targets, which prioritize them in biomedical research ***(2)***. Consequently, profiling membrane proteomes will aid in both understanding basic biological processes and discovering novel targets for therapeutic agents. Membrane proteomics, however, is rendered difficult on several levels.

Notorious difficulties in membrane proteomics include the solubilization, digestion, and separation of membrane proteins. They contain both hydrophilic and hydrophobic domains, which preclude their routine analysis by conventional two-dimensional polyacrylamide gel electrophoresis due to solubilization difficulties. Furthermore, membrane proteins contain stretches of highly hydrophobic transmembrane domains that lack an adequate number of arginine and lysine residues, which are required for in gel digestion with the commonly used endoproteinase trypsin. The sequence coverage of membrane proteins can hence be relatively poor when only this endoproteinase is used.

Some of these problems have been overcome by advances using nongel-based liquid chromatography techniques applying a wide variety of stationary

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and mobile phases and chromatography types *(3)*. Recent separation protocols made use of size-exclusion chromatography (SEC), affinity chromatography (AC), ion-exchange chromatography (IEX) and reversed-phase chromatography (RPC) *(3)*. In the multidimensional protein identification technology (Mud-PIT), for instance, peptide mixtures are loaded onto a biphasic microcapillary column packed with reversed-phase and strong-cation-exchange material. Sequentially, eluted peptides are detected and identified by MS/MS *(4)*. This approach was recently taken a step forward by combining it with methanol-facilitated solubilization and digestion of the protein sample. This combination allowed identification of 1,500-2,500 proteins starting with 100-200 µg of membrane proteins *(5)*.

Another serious, but often underestimated challenge in membrane proteomics is the complexity of a eukaryotic cell. A typical eukaryotic cell contains many different subcellular compartments each with its own subset of membrane proteins. In addition, the subcellular distribution pattern of a given protein might change under different conditions and this might alter its function. Striking example are integral plasma membrane proteins such as the glucose transporter Glut4 in muscle and fat cells *(6)* or the glutamate receptors *(7)* in the brain, which can shuttle between intracellular stores and the plasma membrane under different physiological conditions. Therefore, it is mandatory to characterize these subproteomes separately in order to identify compartment-specific proteins, to describe the physiological state of a tissue under defined conditions, and to gain insight into pathophysiological mechanisms.

Unfortunately, most of the approaches mentioned before do not address this issue or fail to perform sufficiently well in separating the different membraneous compartments *(8)*. There is hence a clear need for the reproducible production of well-characterized membrane protein samples. In this chapter, we provide a robust and efficient protocol to isolate plasma membranes from brain tissue *(9)*. It is based on an aquous polymer two-phase system exploiting the principle of countercurrent distribution. Advantages of this protocol include its ease and efficiency as well as the low costs. Furthermore, two phase systems are very versatile allowing also isolation of substructures of membranes such as caveolae (for further details on membrane partitioning see *[10]*). The protocol can be adapted to other tissues as well.

2. Materials

Due to the strong influence of ions on membrane partitioning in the two-phase systems, double distilled water should be used throughout the experiments.

1. Glass-Teflon homogenizer.
2. Dextran stock solution: Dextran T500 (20% w/w) (*see* **Note 1**).
3. PEG stock solution: PEG 3350 (40% w/w).
4. Tris-H_2SO_4: 200 mM Tris, pH 7.8 adjusted with H_2SO_4.

3. Methods

3.1 Two-Phase Partitioning

All steps of the affinity two-phase partitioning protocol should be performed at 4 °C. Working at room temperature prevents phase separation. The procedure is illustrated in **Fig. 1**. The numbers in **Fig. 1** correspond to the numbered two-phase systems given in **Table 1**, and the letters refer to the phases as indicated in the protocol given below.

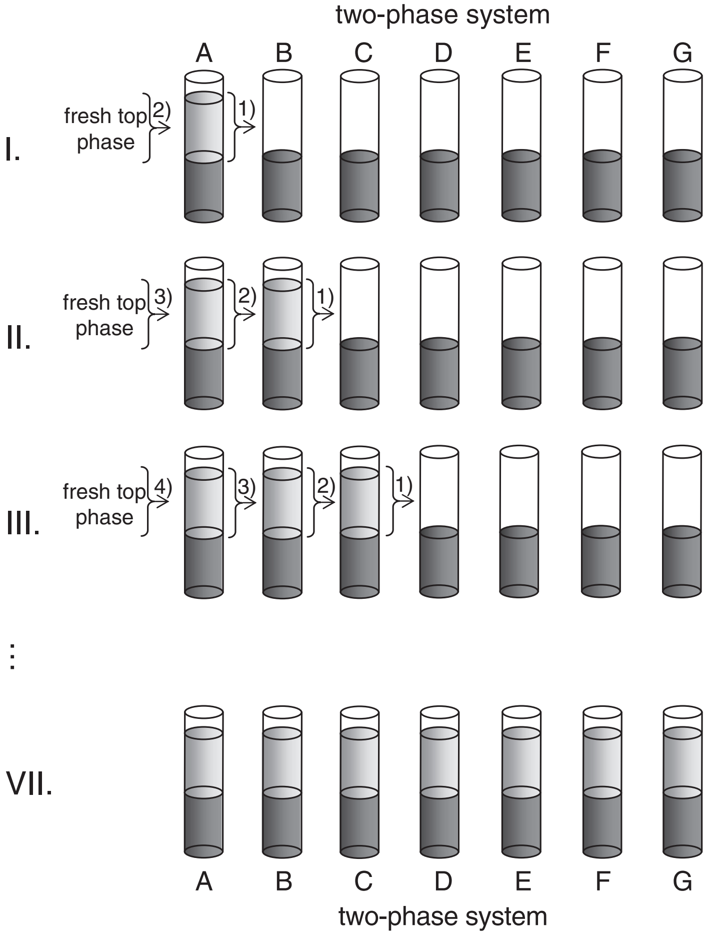
two-phase system
A
B
C
D
E
F
G
I.
fresh top phase
2)
1)
II.
fresh top phase
3)
2)
1)
III.
fresh top phase
4)
3)
2)
1)
⋮
VII.
A
B
C
D
E
F
G
two-phase system

Fig. 1. Scheme of countercurrent distribution. In CD experiments, the top phase of the first two-phase system A is transferred to a fresh bottom phase B and the bottom phase of two-phase system A is reextracted with a fresh top phase. After six iterations, biomaterial is efficiently separated.

Table 1
Composition of Two-Phase Systems

	Two-phase system "A"	Two-phase systems "B-G"
Dextran stock solution	1.035 g	1.035 g
PEG stock solution	0.518 g	0.518 g
Tris-H_2SO_4	0,750 g	0.750 g
Water	0.598 g	0.698 g

1. Prepare seven two-phase systems with the compositions indicated in **Table 1** one day prior to use. Mix them by 20 invertations, vortexing for 10 sec, another 20 invertations and store the mixtures at 4°C overnight. Two-phase systems will form over night with the top phase enriched in PEG and the bottom phase enriched in dextran.
2. On the next day, remove all top phases from two phase systems "B-G" and store them separately.
3. Homogenize 0.100 g brain tissue (*see* **Note 2**) in two-phase system "A" using a glass-teflon homogenizer followed by 45 sec of sonication. Centrifuge at 700 × g for 5 min to accelerate phase separation.
4. Transfer the top phase (Top) of two-phase system "A" onto the bottom-phase (Bot) of two-phase system "B" (TopA -> BotB) (*see* **Note 3**). Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A" (I. in **Fig. 1**). Mix both two phase systems by 20 invertations, vortex for 10 s, then mix again by another 20 invertations. Centrifuge at 700 × g for 5 min to accelerate phase separation.
5. Transfer top phases in the following order (II. in **Fig. 1**): 1) TopB -> BotC; 2) TopA -> BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A." Mix all two-phase systems by 20 invertations, vortexing for 10 sec, and another 20 invertations. Centrifuge at 700 × g for 5 min to accelerate phase separation.
6. Transfer top phases in the following order (III. in **Fig. 1**): (1) TopC -> BotD; (2) TopB -> BotC; (3) Top A -> BotB. Add an equal amount of fresh top-phase (stored in **step 2**) on to bottom-phase "A."
7. Mix all two-phase systems by 20 invertations, vortexing for 10 sec, and another 20 invertations. Centrifuge at 700 × g for 5 min to accelerate phase separation.
8. Transfer top phases in the following order: (1) Top D -> BotE; (2) TopC -> BotD; (3) TopB -> BotC; (4) Top A -> BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A." Mix all two-phase systems by 20 invertations, vortexing for 10 sec, and another 20 invertations. Centrifuge at 700 × g for 5 min to accelerate phase separation.
9. Transfer top phases in the following order: (1) TopE -> BotF; (2) Top D -> BotE; (3) TopC -> BotD; (4) TopB -> BotC; (5) Top A -> BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A". Mix all two-phase systems by 20 invertations, vortexing for 10 sec, and another 20 invertations. Centrifuge at 700 × g for 5 min to accelerate phase separation.

10. Transfer top phases in the following order: (1) TopF -> BotG; (2) TopE -> BotF; (3) Top D -> BotE; (4) TopC -> BotD; (5) TopB -> BotC; 6) Top A -> BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A."
11. Mix all two phase systems by 20 invertations, vortexing for 10 sec, and another 20 invertations. Centrifuge at 700 × g for 5 min to accelerate phase separation. After phase separation you end up with 7 two-phase systems (VII. in **Fig. 1**). Plasma membranes are enriched in top phases "F+G."
12. To proceed further, isolate membranes out of TopG (maximum purity) or from TopG combined with TopF (maximum yield) by diluting the phases 1:10 with water and spin for 1 h at 150,000 × g (*see* **Note 4**).
13. Proteins in the pellet are solubilized by sonication in Laemmli-sample buffer. After incubation for 15 min at 37 °C the sample is separated by SDS-PAGE (*see* Note 5).

4. Notes

1. Dextran can contain up to 10% water and for that reason the 20% stock solution has to be prepared from freeze-dried dextran. For freeze drying, dissolve dextran in distilled water in a plastic dish with a large surface (e.g. Petri dish), freeze it at −80 °C and dry it by sublimating the water under vacuum. Store the freeze-dried dextran in closed plastic tubes sealed tightly with parafilm at −20 °C. Let it come to room temperature before opening to protect it from humidity.
2. The protocol can also be applied to other tissues. Applicants should take care that the tissue is thoroughly homogenized (e.g., sceletal muscle and heart tissue are much harder to homogenize than brain tissue) or the yield of plasma membranes will decrease. Altering the polymer concentration or the buffer conditions might be considered to optimize the protocol for the fractionation of other tissues (for review see Schindler and Nothwang, 2006)
3. In two-phase systems, interphases are always assigned to the bottom phase.
4. The amount of peripheral membrane proteins in the pellet can be reduced by extractions with Na_2CO_3. The procedure solubilizes peripheral membrane proteins whereas integral proteins remain in the pellet **(10).** For this purpose, resuspend the pellet in ice cold Na_2CO_3 (0.2 M), incubate for 15 min on ice and pellet the membranes by ultracentrifugation at 233,000 × g for 1 h. This step might be repeated twice.
5. Proteins can also be solubilized in different buffers, depending on further processing (e.g., 16-BAC-SDS-PAGE, BN-PAGE, immunoprecipitation)

Acknowledgments

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Subcellular Fractionation of Small Sample Amounts

Hans Gerd Nothwang, Isabelle Guillemin, and Jens Schindler

1. Introduction

A central issue in proteomics is the generation of qualitative and quantitative protein inventories for a given tissue, organ, or organism. In many eukaryotes, this amounts to a formidable task. Mammals may express more than 200,000 different transcripts with a quarter of them encoding proteins ***(1)***. In addition, there exist several hundred different ***(2)*** posttranslational modifications, increasing the number of different protein species by an estimated factor of 10, compared to the number of different mRNAs ***(3,4)***. Consequently, higher organism will display more than 1,000,000 different proteins ***(5)***. Considering as a lower, very conservative limit, the expression of 5–10% of the transcriptome in an organ, about 50,000–100,000 different protein species will be present in such a sample. Even in a single cell, > 10,000 different protein species likely exist. This number easily exceeds the potential of any known proteomic approach. Another important issue in protein analysis is the high range of protein abundance. Protein concentrations likely vary between 10^6–10^{10}, depending on the tissue analyzed ***(6,7)***. This renders the identification of low abundant proteins impossible in the bulk of total protein extracts.

To overcome these hurdles, many novel sample fraction approaches have been developed. These include SDS-PAGE combined with liquid chromatography coupled mass spectrometry or multidimensional liquid chromatography techniques ***(8,9)***. However, when aiming at proteomics as a bottom-up process to understand a biological system, most of these approaches fall short by neglecting the importance of the compartmentalized organization of any living cell. A cell is highly structured and organized. Most, if not all proteins have precise subcellular locations and mistargeting might have severe pathophysiological consequences. It matters, whether a transcription factor such as NF-kappaB is in the cytosol

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or in the nucleus. Furthermore, posttranslational modifications depend on the localization of a protein. A striking example is the addition and modification of sugar residues during the passage of plasma membrane proteins through the biosynthetic secretory pathway. Therefore, everything in proteomics makes only sense in the light of subcellular organization and this information should be retained during sample preparation. Here, we present a subcellular fractionation protocol, developed for minute amounts of tissue or cell cultures ***(10)***. The protocol is based on hypotonic cell lysis, followed by differential centrifugation. It yields three fractions, a nuclei-enriched fraction, a combined membrane and organelle enriched fraction, and a cytoplasmic fraction.

2. Materials

1. CLB: 10 mM HEPES, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5 mM EDTA,1 mM$CaCl_2$, 0.5 mM $MgCl_2$, and Complete protease inhibitor (Roche, Penzberg, Germany).
2. TSE: 10 mM Tris, 300 mM sucrose, 1 mM EDTA, 0.1% IGEPAL-CA 630 (v/v), pH 7.5 (*see* **Note 1**).
3. 2.5 M sucrose.
4. PBS: 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4.
5. Motorized Teflon/glass homogenizer.
6. Table top centrifuge (e.g. 5417R, Eppendorf, Hamburg, Germany).
7. Beckman 70Ti rotor and Beckman ultracentrifuge (Beckman Coulter, Krefeld, Germany).
8. Micro-BCA assay (Pierce, Bonn, Germany).
9. 2-D gel clean-up kit (GE Healthcare, Freiburg, Germany).
10. Rehydration buffer: 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB-3-10, 40 mM Tris, trace of bromophenol blue.

3. Methods

3.1. Fractionation of Cultures Cells

All steps of the protocol should be performed on ice. The procedure is illustrated in **Fig. 1**.

1. Harvest ~8 × 10^6 cells of a monolayer culture (75 cm^2) by trypsination.
2. Incubate the cells in 1 ml of CLB for 5 min on ice.
3. Homogenize the cells with 50 strokes in a motor-driven homogenizer at 250 rpm. Keep the sample on ice.
4. Add 100 µl of 2.5 M sucrose to the sample to restore isotonic condition.
5. Centrifuge the sample at 6,300 × g for 5 min at 4°C.
6. Transfer the supernatant to a new tube. It will be processed in **step 11**.
7. Homogenize the pellet in 500 µl of CLB /0.25 M sucrose with a 1 ml pipette tip.
8. Centrifuge the sample at 6,300 × g for 5 min at 4°C.
9. Combine the supernatant with the supernatant of **step 6**.

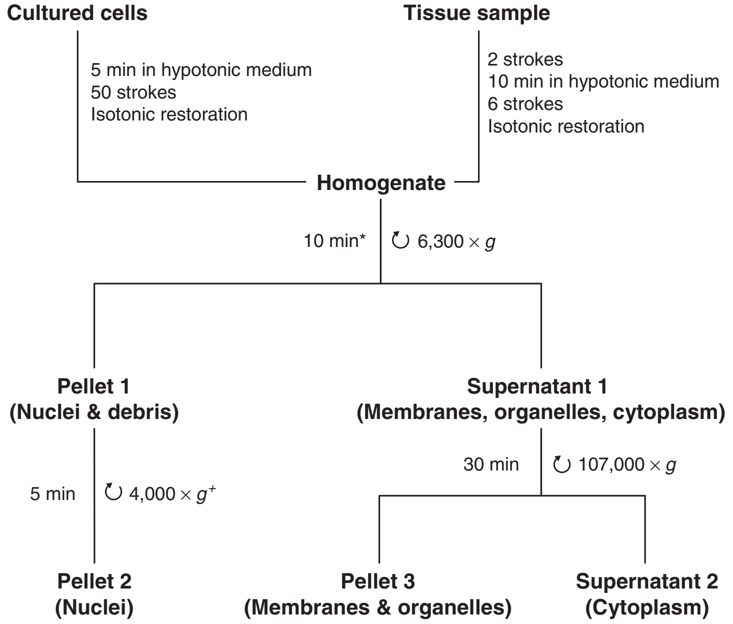
Cultured cells
Tissue sample
5 min in hypotonic medium
50 strokes
Isotonic restoration
2 strokes
10 min in hypotonic medium
6 strokes
Isotonic restoration
Homogenate
10 min*
6,300 × g
Pellet 1
(Nuclei & debris)
Supernatant 1
(Membranes, organelles, cytoplasm)
5 min
4,000 × g+
30 min
107,000 × g
Pellet 2
(Nuclei)
Pellet 3
(Membranes & organelles)
Supernatant 2
(Cytoplasm)

Fig. 1. Schematic illustration of the subcellular fractionation protocol. Conditions of homogenization depend on the starting material, that is, either cultured cells or tissue. After homogenization, two rounds of differential centrifugation with increasing centrifugal force yield three final fractions. * for cultured cells, the first round of centrifugation lasted only 5 min; + for freshly prepared tissue, nuclei should be pelleted at only 1000 × g to prevent cosedimentation with other subcellular compartments.

10. Homogenize the pellet in 1 ml TSE with 30 strokes in a motor-driven homogenizer at 250 rpm.
11. Centrifuge the suspension at 4,000 × g for 5 min at 4°C (see **Note 2**).
12. Discard the supernatant and repeat **steps 10** and **11** until the supernatant remains clear. Resuspend the final pellet in 200 µl of TSE and store it at –20°C until further usage. This suspension represents the nuclei enriched fraction.
13. Sediment the combined supernatants from step 6 and 9 at 107,000 × g for 30 min at 4°C min using a Beckman 70Ti rotor (or equivalent) in a Beckman ultracentrifuge (*see* **Note 3**).
14. Transfer the supernatant to a new tube and store it at –20 °C until further usage. It represents the cytosolic fraction.
15. Resuspend the pellet in 80 µl of PBS. It represents the membrane and organelle enriched fraction. Store it at –20°C until further usage.

3.2. Fractionation of Tissue

1. Transfer 500 mg of frozen tissue (e.g. brain) to 1 ml of ice cold CLB.
2. Prehomogenize the tissue by 2 strokes in a glass/Teflon homogenizer on ice.

3. Incubate the pre-homogenate on ice for 10 min.
4. Homogenize the prehomogenate by 6 strokes in a motorized glass/Teflon homogenizer at 250 rpm (*see* **Note 4**).
5. Add 100 µl of 2.5 M sucrose to restore isotonic condition.
6. Centrifuge the sample at 6,300 × g for 10 min at 4°C (*see* **Notes 5** and **6**).
7. Transfer the supernatant to a new tube. It will be processed in **step 11**.
8. Homogenize the pellet in 500 µl of CLB /0.25 M sucrose with a 1 ml pipette tip.
9. Centrifuge the sample at 6,300 × g for 5 min at 4°C
10. Combine the supernatant with the supernatant of **step 7**.
11. Homogenize the pellet in 1 ml of TSE by 30 strokes in a motor-driven homogenizer at 250 rpm.
12. Centrifuge the sample at 4,000 × g for 5 min at 4°C (*see* **Note 2**).
13. Discard the supernatant and repeat **steps 7** and **8** until the supernatant remains clear.
14. Resuspend the final pellet in 200 µl of TSE and store it at −20°C until further usage. This suspension represents the nuclei enriched fraction.
15. Sediment the supernatant from steps 7 and 10 at 107,000 × g for 30 min at 4°C min using a Beckman Ti70 rotor (or equivalent) in a Beckman ultracentrifuge (*see* **Note 3**).
16. Transfer the supernatant to a new tube and store it at −20°C until further usage. It represents the cytosolic fraction.
17. Resuspend the pellet in 80 µl of PBS. It represents the membrane and organelle enriched fraction. Store it at −20°C until further use.

3.3. Protein Quantification and Clean-Up

The following procedure is applied to analyze the samples by conventional two-dimensional gel electrophoresis.

1. Determine the protein concentration of the cytosolic fraction by the Micro BCA Protein Assay Reagent Kit according to the manurfacturer's instructions. Use 1, 2, and 5 µl of the sample and BSA as standard.
2. If required for further analysis, concentrate the sample by acetone precipitation, as outlined in **steps 3 to 8.**
3. Add 4 times the sample volume of cold (−20°C) acetone to the sample.
4. Mix well and incubate at −20°C for 1 h.
5. Centrifuge the sample 10 min at 14,000 rpm.
6. Decant the supernatant and remove all remaining liquids carefully with a pipette tip.
7. Allow the acetone to evaporate from the open tube for 30 min (*see* **Note 7**).
8. Resuspend the pellet in the appropriate volume of the desired buffer (e.g., rehydration buffer).
9. Clean-up nuclear and membrane & organelle fractions using a clean-up kit (e.g., 2-D clean up kit, GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions.
10. Dissolve the resulting pellets in 200 µl rehydration buffer (*see* **Note 8**).

11. Estimate the protein content of the nuclei and of the membrane and organelles enriched fraction by comparing an aliquot of these fractions with a dilution series of the cytosolic proteins quantified in **step 1,** after separation in a SDS-PAGE and staining with Coomassie or fluorescent dyes (*see* **Note 9**).

4. Notes

1. IGEPAL-CA 630 is identical to Nonidet P40 substitute.
2. This washing step purifies the nuclear pellet by removing nonnuclear membrane vesicles. Omitting this step will increase the yield but will reduce the purity of the fraction in parallel.
3. Alternatively, this step can be performed for 150 min at 14,000 rpm (corresponding to ~21,000 × g) at 4°C in a tabletop centrifuge. This by-passes the requirement of an ultracentriguge without affecting yield or purity.
4. Cultured cells require a higher number of strokes compared to tissue samples, as their cytoskeleton differs from the cytoskeleton in brain tissue. Tissues such as heart, liver or placenta will also need harsher conditions for homogenization.
5. When fresh tissue is used, centrifuge only at 3,000 rpm.
6. The prolonged centrifugation time compared to cultured cells results in an increased recovery of nuclear marker proteins.
7. Do not overdry, as the samples may not dissolve properly afterward.
8. This buffer with non-ionic detergents is required if samples will be separated by isoelectric focusing. for other downstream applications, an SDS containing buffer might be used. This will improve the resuspension of the pellets. Due to the presence of thiourea in the rehydration buffer, most colorimetric quantification methods will not work.

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Nondenaturing Polyacrylamide Gel Electrophoresis of Proteins

John M. Walker

1. Introduction

SDS-PAGE (Chapter 21) is probably the most commonly used gel electrophoretic system for analyzing proteins. However, it should be stressed that this method separates denatured protein. Sometimes one needs to analyze native, nondenatured proteins, particularly if wanting to identify a protein in the gel by its biological activity (for example, enzyme activity, receptor binding, antibody binding, and so on). On such occasions it is necessary to use a nondenaturing system such as described in this chapter. For example, when purifying an enzyme, a single major band on a gel would suggest a pure enzyme. However this band could still be a contaminant; the enzyme could be present as a weaker (even nonstaining) band on the same gel. Only by showing that the major band had enzyme activity would you be convinced that this band corresponded to your enzyme. The method described here is based on the gel system first described by Davis ***(1)***. To enhance resolution a stacking gel can be included (*see* Chapter 21 for the theory behind the stacking gel system).

2. Materials

1. Stock acrylamide solution: 30 g acrylamide, 0.8 g *bis*-acrylamide. Make up to 100 mL in distilled water and filter. Stable at 4°C for months (*see* **Note 1**). **Care: Acrylamide Monomer Is a Neurotoxin**. Take care in handling acrylamide (wear gloves) and avoid breathing in acrylamide dust when weighing out.
2. Separating gel buffer: 1.5 *M* Tris-HCl, pH 8.8.
3. Stacking gel buffer: 0.5 *M* Tris-HCl, pH 6.8.
4. 10% Ammonium persulfate in water.
5. *N,N,N ,N* -tetramethylethylenediamine (TEMED).

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6. Sample buffer (5X). Mix the following:
   a. 15.5 mL of 1 *M* Tris-HCl pH 6.8;
   b. 2.5 mL of a 1% solution of bromophenol blue;
   c. 7 mL of water; and
   d. 25 mL of glycerol.

   Solid samples can be dissolved directly in IX sample buffer. Samples already in solution should be diluted accordingly with 5X sample buffer to give a solution that is 1X sample buffer. Do not use protein solutions that are in a strong buffer that is not near to pH 6.8 as it is important that the sample is at the correct pH. For these samples it will be necessary to dialyze against 1X sample buffer.
7. Electrophoresis buffer: Dissolve 3.0 g of Tris base and 14.4 g of glycine in water and adjust the volume to 1 L. The final pH should be 8.3.
8. Protein stain: 0.25 g Coomassie brilliant blue R250 (or PAGE blue 83), 125 mL methanol, 25 mL glacial acetic acid, and 100 mL water. Dissolve the dye in the methanol component first, then add the acid and water. Dye solubility is a problem if a different order is used. Filter the solution if you are concerned about dye solubility. For best results do not reuse the stain.
9. Destaining solution: 100 mL methanol, 100 mL glacial acetic acid, and 800 mL water.
10. A microsyringe for loading samples.

3. Method

1. Set up the gel cassette.
2. To prepare the separating gel (*see* **Note 2**) mix the following in a Buchner flask: 7.5 mL stock acrylamide solution, 7.5 mL separating gel buffer, 14.85 mL water, and 150 mL 10% ammonium persulfate.

   "Degas" this solution under vacuum for about 30 s. This degassing step is necessary to remove dissolved air from the solution, since oxygen can inhibit the polymerization step. Also, if the solution has not been degassed to some extent, bubbles can form in the gel during polymerization, which will ruin the gel. Bubble formation is more of a problem in the higher percentage gels where more heat is liberated during polymerization.
3. Add 15 µL of TEMED and gently swirl the flask to ensure even mixing. The addition of TEMED will initiate the polymerization reaction, and although it will take about 20 min for the gel to set, this time can vary depending on room temperature, so it is advisable to work fairly quickly at this stage.
4. Using a Pasteur (or larger) pipet, transfer the separating gel mixture to the gel cassette by running the solution carefully down one edge between the glass plates. Continue adding this solution until it reaches a position 1 cm from the bottom of the sample loading comb.
5. To ensure that the gel sets with a smooth surface, *very* carefully run distilled water down one edge into the cassette using a Pasteur pipet. Because of the great difference in density between the water and the gel solution, the water will

spread across the surface of the gel without serious mixing. Continue adding water until a layer about 2 mm exists on top of the gel solution.

6. The gel can now be left to set. When set, a very clear refractive index change can be seen between the polymerized gel and overlaying water.
7. While the separating gel is setting, prepare the following stacking gel solution. Mix the following quantities in a Buchner flask: 1.5 mL stock acrylamide solution, 3.0 mL stacking gel buffer, 7.4 mL water, and 100 µL 10% ammonium persulfate. Degas this solution as before.
8. When the separating gel has set, pour off the overlaying water. Add 15 µL of TEMED to the stacking gel solution and use some (2 mL) of this solution to wash the surface of the polymerized gel. Discard this wash, then add the stacking gel solution to the gel cassette until the solution reaches the cutaway edge of the gel plate. Place the well-forming comb into this solution and leave to set. This will take about 30 min. Refractive index changes around the comb indicate that the gel has set. It is useful at this stage to mark the positions of the bottoms of the wells on the glass plates with a marker pen.
9. Carefully remove the comb from the stacking gel, remove any spacer from the bottom of the gel cassette, and assemble the cassette in the electrophoresis tank. Fill the top reservoir with electrophoresis buffer ensuring that the buffer fully fills the sample loading wells, and look for any leaks from the top tank. If there are no leaks, fill the bottom tank with electrophoresis buffer, then tilt the apparatus to dispel any bubbles caught under the gel.
10. Samples can now be loaded onto the gel. Place the syringe needle through the buffer and locate it just above the bottom of the well. Slowly deliver the sample (5–20 µL) into the well. The dense sample solvent ensures that the sample settles to the bottom of the loading well. Continue in this way to fill all the wells with unknowns or standards, and record the samples loaded.
11. The power pack is now connected to the apparatus and a current of 20–25 mA passed through the gel (constant current) (*see* **Note 3**). Ensure that the electrodes are arranged so that the proteins are running to the anode (*see* **Note 4**). In the first few minutes the samples will be seen to concentrate as a sharp band as it moves through the stacking gel. (It is actually the bromophenol blue that one is observing, not the protein but, of course, the protein is stacking in the same way.) Continue electrophoresis until the bromophenol blue reaches the bottom of the gel. This will usually take about 3 h. Electrophoresis can now be stopped and the gel removed from the cassette. Remove the stacking gel and immerse the separating gel in stain solution, or proceed to step 13 if you wish to detect enzyme activity (*see* **Notes 5** and **6**).
12. Staining should be carried out, with shaking, for a minimum of 2 h and preferably overnight. When the stain is replaced with destain, stronger bands will be immediately apparent and weaker bands will appear as the gel destains. Destaining can be speeded up by using a foam bung, such as those used in microbiological flasks. Place the bung in the destain and squeeze it a few times to expel air bubbles and ensure the bung is fully wetted. The bung rapidly absorbs dye, thus speeding up the destaining process.

13. If proteins are to be detected by their biological activity, duplicate samples should be run. One set of samples should be stained for protein and the other set for activity. Most commonly one would be looking for enzyme activity in the gel. This is achieved by washing the gel in an appropriate enzyme substrate solution that results in a colored product appearing in the gel at the site of the enzyme activity (*see* **Note 7**).

4. Notes

1. The stock acrylamide used here is the same as used for SDS gels (*see* Chapter 21) and may already be availabe in your laboratory.
2. The system described here is for a 7.5% acrylamide gel, which was originally described for the separation of serum proteins ***(1)***. Since separation in this system depends on both the native charge on the protein and separation according to size owing to frictional drag as the proteins move through the gel, it is not possible to predict the electrophoretic behavior of a given protein the way that one can on an SDS gel, where separation is based on size alone. A 7.5% gel is a good starting point for unknown proteins. Proteins of mol wt >100,000 should be separated in 3–5% gels. Gels in the range 5–10% will separate proteins in the range 20,000–150,000, and 10–15% gels will separate proteins in the range 10,000–80,000. The separation of smaller polypeptides is described in Chapter 23. To alter the acrylamide concentration, adjust the volume of stock acrylamide solution in **Subheading 3., step 2** accordingly, and increase/decrease the water component to allow for the change in volume. For example, to make a 5% gel change the stock acrylamide to 5 mL and increase the water to 17.35 mL. The final volume is still 30 mL, so 5 mL of the 30% stock acrylamide solution has been diluted in 30 mL to give a 5% acrylamide solution.
3. Because one is separating native proteins, it is important that the gel does not heat up too much, since this could denature the protein in the gel. It is advisable therefore to run the gel in the cold room, or to circulate the buffer through a cooling coil in ice. (Many gel apparatus are designed such that the electrode buffer cools the gel plates.) If heating is thought to be a problem it is also worthwhile to try running the gel at a lower current for a longer time.
4. This separating gel system is run at pH 8.8. At this pH most proteins will have a negative charge and will run to the anode. However, it must be noted that any basic proteins will migrate in the opposite direction and will be lost from the gel. Basic proteins are best analyzed under acid conditions, as described in Chapters 27 and 28.
5. It is important to note that concentration in the stacking gel may cause aggregation and precipitation of proteins. Also, the pH of the stacking gel (pH

6.8) may affect the activity of the protein of interest. If this is thought to be a problem (e.g., the protein cannot be detected on the gel), prepare the gel without a stacking gel. Resolution of proteins will not be quite so good, but will be sufficient for most uses.

6. If the buffer system described here is unsuitable (e.g., the protein of interest does not electrophorese into the gel because it has the incorrect charge, or precipitates in the buffer, or the buffer is incompatible with your detection system) then one can try different buffer systems (without a stacking gel). A comprehensive list of alternative buffer systems has been published ***(2)***.
7. The most convenient substrates for detecting enzymes in gels are small molecules that freely diffuse into the gel and are converted by the enzyme to a colored orfluorescent product within the gel. However, for many enzymes such convenient substrates do not exist, and it is necessary to design a linked assay where one includes an enzyme together with the substrate such that the products of the enzymatic reaction of interest is converted to a detectable product by the enzyme included with the substrate. Such linked assays may require the use of up to two or three enzymes and substrates to produce a detectable product. In these cases the product is usually formed on the surface of the gel because the coupling enzymes cannot easily diffuse into the gel. In this case the zymogram technique is used where the substrate mix is added to a cooled (but not solidified) solution of agarose (1%) in the appropriate buffer. This is quickly poured over the solid gel where it quickly sets on the gel. The product of the enzyme assay is therefore formed at the gel–gel interface and does not get washed away. A number of review articles have been published which described methods for detecting enzymes in gels ***(3–7)***. A very useful list also appears as an appendix in **ref. *8***.

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SDS Polyacrylamide Gel Electrophoresis of Proteins

John M. Walker

1. Introduction

SDS-PAGE is the most widely used method for qualitatively analyzing protein mixtures. It is particularly useful for monitoring protein purification, and because the method is based on the separation of proteins according to size, the method can also be used to determine the relative molecular mass of proteins (*see* **Note 14**).

1.1. Formation of Polyacrylamide Gels

Crosslinked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of *N,N′* -methylene-*bis*-acrylamide (normally referred to as “*bis*-acrylamide”) (**Fig. 1**). Note that *bis*-acrylamide is essentially two acrylamide molecules linked by a methylene group and is used as a crosslinking agent. Acrylamide monomer is polymerized in a head-to-tail fashion into long chains, and occasionally a *bis*-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way, a crosslinked matrix of fairly well-defined structure is formed (**Fig. 1**). The polymerization of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulfate and the base *N,N,N′,N′*-tetramethylenediamine (TEMED). TEMED catalyzes the decomposition of the persulfate ion to give a free radical (i.e., a molecule with an unpaired electron):

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{-\bullet} \qquad (1)$$

If this free radical is represented as $R^{\bullet}$ (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule, then the polymerization can be represented as follows:

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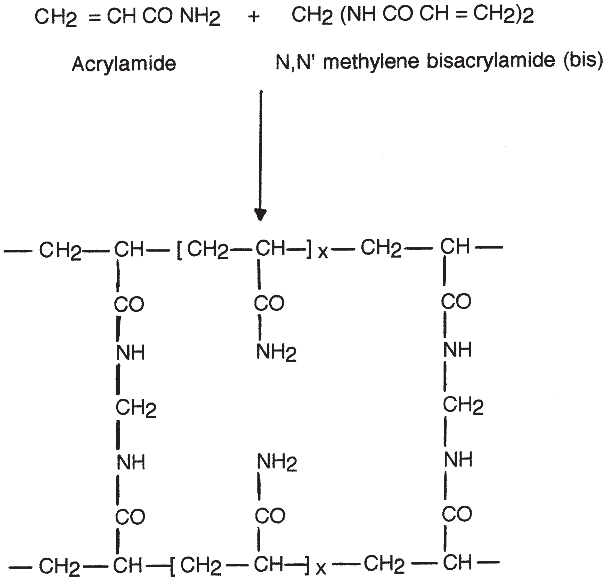
CH2 = CH CO NH2 + CH2 (NH CO CH = CH2)2
Acrylamide
N,N' methylene bisacrylamide (bis)
— CH2— CH— [CH2— CH—] x— CH2— CH —
CO
NH
CH2
NH
CO
CO
NH2
NH2
CO
— CH2— CH—[CH2— CH—] x —CH2 — CH—

Fig. 1. Polymerization of acrylamide.

$$\begin{aligned}R^\bullet + M &\rightarrow RM^\bullet\\ RM^\bullet + M &\rightarrow RMM^\bullet\\ RMM^\bullet + M &\rightarrow RMMM^\bullet\text{, and so forth}\end{aligned} \tag{2}$$

In this way, long chains of acrylamide are built up, being crosslinked by the introduction of the occasional *bis*-acrylamide molecule into the growing chain. Oxygen "mops up" free radicals, and therefore the gel mixture is normally degassed (the solutions are briefly placed under vacuum to remove loosely dissolved oxygen) prior to addition of the catalyst.

1.2. The Use of Stacking Gels

For both SDS and buffer gels samples may be applied directly to the top of the gel in which protein separation is to occur (the separating gel). However, in these cases, the sharpness of the protein bands produced in the gel is limited by the size (volume) of the sample applied to the gel. Basically the separated bands will be as broad (or broader, owing to diffusion) as the sample band applied to the gel. For some work, this may be acceptable, but most workers require better resolution than this. This can be achieved by polymerizing a short stacking gel on top of the separating gel. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel, thus giving sharper protein bands in the separating gel. This modification allows relatively large sample volumes to be applied to the gel without any loss of resolution. The stacking gel has a very large pore size (4% acrylamide) which allows the proteins to move freely and concentrate, or stack under the effect of the electric field. Sample concentration is produced by isotachophoresis of the sample in the stacking gel. The band-sharpening effect (isotachophoresis) relies on the fact that the negatively charged glycinate ions (in the reservoir buffer) have a lower electrophoretic mobility than the protein–SDS complexes. which in turn, have lower mobility than the Cl^- ions if they are in a region of higher field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The result is that the three species of interest adjust their concentrations so that $[Cl^-]$ > [protein-SDS] > [glycinate]. There are only a small quantity of protein–SDS complexes, so they concentrate in a very tight band between the glycinate and Cl^- ion boundaries. Once the glycinate reaches the separating gel, it becomes more fully ionized in the higher pH environment and its mobility increases. (The pH of the stacking gel is 6.8 and that of the separating gel is 8.8.) Thus, the interface between glycinate and the Cl^- ions leaves behind the protein–SDS complexes, which are left to electrophorese at their own rates. A more detailed description of the theory of isotachophoresis and electrophoresis generally is given in **ref.** ***1***.

1.3. SDS-PAGE

Samples to be run on SDS-PAGE are first boiled for 5 min in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces any disulfide bridges present that are holding together the protein tertiary structure. SDS (CH_3-$[CH_2]_{10}$ - CH_2OSO_3-Na^+) is an anionic detergent and binds strongly to, and denatures, the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the SDS molecules. The sample buffer also contains an ionizable tracking dye usually bromophenol blue that allows the electrophoretic run to be monitored, and sucrose or glycerol which gives the sample solution density, thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well. When the main separating gel has been poured between the glass plates and allowed to set, a shorter stacking gel is poured on top of the separating gel, and it is into this gel that the wells are formed and the proteins loaded. Once all samples are loaded, a current is passed through the gel. Once the protein samples have passed through the stacking gel and have entered the separating gel, the negatively charged protein–SDS complexes continue to move toward the anode, and because they have the same charge per unit length they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply, the smaller the protein, the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance owing to the sieving effect of the gel. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front. When the dye reaches the bottom of the gel the current is turned off and the gel is removed from between the glass plates, shaken in an appropriate stain solution (usually Coomassie brilliant blue) for a few hours, and then washed in destain solution overnight. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background. A typical large format gel would take about 1 h to prepare and set, 3 h to run at 30 mA, and have a staining time of 2–3 h with an overnight destain. Minigels (e.g., Bio-Rad minigel) run at 200 V. Constant voltage can run in about 40 min, and require only 1 h staining. Most bands can be seen within 1 h of destaining. Vertical slab gels are invariably run since this allows up to 20 different samples to be loaded onto a single gel.

2. Materials

1. Stock acrylamide solution: 30% acrylamide, 0.8% *bis*-acrylamide. Filter through Whatman No. 1 filter paper and store at 4°C (*see* **Note 1**).
2. Buffers:
   a. 1.875 *M* Tris-HCl, pH 8.8.
   b. 0.6 *M* Tris-HCl, pH 6.8.
3. 10% Ammonium persulfate. Make fresh.
4. 10% SDS (*see* **Note 2**).
5. TEMED.
6. Electrophoresis buffer: Tris (12 g), glycine (57.6 g), and SDS (2.0 g). Make up to 2 L with water. No pH adjustment is necessary.
7. Sample buffer (*see* **Notes 3** and **4**):

0.6 *M* Tris-HCl, pH 6.8	5.0 mL
SDS	0.5 g
Sucrose	5.0 g
β-Mercaptoethanol	0.25 mL
Bromophenol blue, 0.5% stock	5.0 mL

   Make up to 50 mL with distilled water.
8. Protein stain: 0.1% Coomassie brilliant blue R250 in 50% methanol, 10% glacial acetic acid. Dissolve the dye in the methanol and water component first, and then add the acetic acid. Filter the final solution through Whatman No. 1 filter paper if necessary.

9. Destain: 10% methanol, 7% glacial acetic acid.
10. Microsyringe for loading samples. Micropipet tips that are drawn out to give a fine tip are also commercially available.

3. Method

The system of buffers used in the gel system described below is that of Laemmli ***(2)***.

1. Samples to be run are first denatured in sample buffer by heating to 95–100°C for 5 min (*see* **Note 3**).
2. Clean the internal surfaces of the gel plates with detergent or methylated spirits, dry, then join the gel plates together to form the cassette, and clamp it in a vertical position. The exact manner of forming the cassette will depend on the type of design being used.
3. Mix the following in a 250-mL Buchner flask (*see* **Note 5**):

	For 15% gels	For 10% gels
1.875 *M* Tris-HCl, pH 8.8	8.0 mL	8.0 mL
Water	11.4 mL	18.1 mL
Stock acrylamide	20.0 mL	13.3 mL
10% SDS	0.4 mL	0.4 mL
Ammonium persulfate (10%)	0.2 mL	0.2 mL

4. "Degas" this solution under vacuum for about 30 s. Some frothing will be observed, and one should not worry if some of the froth is lost down the vacuum tube: you are only losing a very small amount of liquid (*see* **Note 6**).
5. Add 14 µL of TEMED, and gently swirl the flask to ensure even mixing. The addition of TEMED will initiate the polymerization reaction and although it will take about 15 min for the gel to set, this time can vary depending on room temperature, so it is advisable to work fairly quickly at this stage.
6. Using a Pasteur (or larger) pipet transfer this separating gel mixture to the gel cassette by running the solution carefully down one edge between the glass plates. Continue adding this solution until it reaches a position 1 cm from the bottom of the comb that will form the loading wells. Once this is completed, you will find excess gel solution remaining in your flask. Dispose of this in an appropriate waste container **not** down the sink.
7. To ensure that the gel sets with a smooth surface **very carefully** run distilled water down one edge into the cassette using a Pasteur pipet. Because of the great difference in density between the water and the gel solution the water will spread across the surface of the gel without serious mixing. Continue adding water until a layer of about 2 mm exists on top of the gel solution (*see* **Notes 7** and **8**).
8. The gel can now be left to set. As the gel sets, heat is evolved and can be detected by carefully touching the gel plates. When set, a very clear refractive index change can be seen between the polymerized gel and overlaying water.

9. While the separating gel is setting prepare the following stacking gel (4°C) solution. Mix the following in a 100-mL Buchner flask (*see* **Notes 8** and **9**):

0.6 *M* Tris-HCl, pH 6.8	1.0 mL
Stock acrylamide	1.35 mL
Water	7.5 mL
10% SDS	0.1 mL
Ammonium persulfate (10%)	0.05 mL

   Degas this solution as before.
10. When the separating gel has set, pour off the overlaying water. Add 14 µL of TEMED to the stacking gel solution and use some (~2 mL) of this solution to wash the surface of the polymerized gel. Discard this wash, and then add the stacking gel solution to the gel cassette until the solution reaches the cutaway edge of the gel plate. Place the well-forming comb into this solution, and leave to set. This will take about 20 min. Refractive index changes around the comb indicate that the gel has set. It is useful at this stage to mark the positions of the bottoms of the wells on the glass plates with a marker pen to facilitate loading of the samples (*see* also **Note 9**).
11. Carefully remove the comb from the stacking gel, and then rinse out any non-polymerized acrylamide solution from the wells using electrophoresis buffer. Remove any spacer from the bottom of the gel cassette, and assemble the cassette in the electrophoresis tank. Fill the top reservoir with electrophoresis buffer, and look for any leaks from the top tank. If there are no leaks fill the bottom tank with electrophoresis buffer, and then tilt the apparatus to dispel any bubbles caught under the gel.
12. Samples can now be loaded onto the gel. Place the syringe needle through the buffer and locate it just above the bottom of the well. Slowly deliver the sample into the well. Five- to 10-µL samples are appropriate for most gels. The dense sample buffer ensures that the sample settles to the bottom of the loading well (*see* **Note 10**). Continue in this way to fill all the wells with unknowns or standards, and record the samples loaded.
13. Connect the power pack to the apparatus, and pass a current of 30 mA through the gel (constant current) for large format gels, or 200 V (constant voltage) for minigels (Bio-Rad). Ensure your electrodes have correct polarity: all proteins will travel to the anode (+). In the first few minutes, the samples will be seen to concentrate as a sharp band as it moves through the stacking gel. (It is actually the bromophenol blue that one is observing not the protein, but of course the protein is stacking in the same way.) Continue electrophoresis until the bromophenol blue reaches the bottom of the gel. This will take 2.5–3.0 h for large format gels (16 cm × 16 cm) and about 40 min for minigels (10 cm × 7 cm) (*see* **Note 11**).
14. Dismantle the gel apparatus, pry open the gel plates, remove the gel, discard the stacking gel, and place the separating gel in stain solution.
15. Staining should be carried out with shaking, for a minimum of 2 h. When the stain is replaced with destain, stronger bands will be immediately apparent, and weaker bands will appear as the gel destains (*see* **Notes 12** and **13**).

4. Notes

1. Acrylamide is a potential neurotoxin and should be treated with great care. Its effects are cumulative, and therefore, regular users are at greatest risk. In particular, take care when weighing out acrylamide. Do this in a fume hood, and wear an appropriate face mask.
2. SDS come out of solution at low temperature, and this can even occur in a relatively cold laboratory. If this happens, simply warm up the bottle in a water bath. Store at room temperature.
3. Solid samples can be dissolved directly in sample buffer. Pure proteins or simple mixtures should be dissolved at 1–0.5 mg/mL. For more complex samples suitable concentrations must be determined by trial and error. For samples already in solution dilute them with an equal volume of double-strength sample buffer. Do not use protein solutions that are in a strong buffer, that is, not near pH 6.5, since it is important that the sample be at the correct pH. For these samples, it will be necessary to dialyze them first. Should the sample solvent turn from blue to yellow, this is a clear indication that your sample is acidic.
4. The β-mercaptoethanol is essential for disrupting disulfide bridges in proteins. However, exposure to oxygen in the air means that the reducing power of β-mercaptoethanol in the sample buffer decreases with time. Every couple of weeks, therefore, mercaptoethanol should be added to the stock solution or the solution remade. Similarly protein samples that have been prepared in sample buffer and stored frozen should, before being rerun at a later date, have further mercaptoethanol added.
5. Typically, the separating gel used by most workers is a 15% polyacrylamide gel. This give a gel of a certain pore size in which proteins of relative molecular mass (M_r) 10,000 move through the gel relatively unhindered, whereas proteins of 100,000 can only just enter the pores of this gel. Gels of 15% polyacrylamide are therefore useful for separating proteins in the range of 100,000–10,000. However, a protein of 150,000 for example, would be unable to enter a 15% gel. In this case, a larger-pored gel (e.g., a 10% or even 7.5% gel) would be used so that the protein could now enter the gel, and be stained and identified. It is obvious, therefore, that the choice of gel to be used depends on the size of the protein being studied. If proteins covering a wide range of mol-wt values need to be separated, then the use of a gradient gel is more appropriate (*see* Chapter 32).
6. Degassing helps prevent oxygen in the solution from "mopping up" free radicals and inhibiting polymerization although this problem could be overcome by the alternative approach of increasing the concentration of catalyst. However, the polymerization process is an exothermic one. For 15% gels, the heat liberated can result in the formation of small air bubbles

in the gel (this is not usually a problem for gels of 10% or less where much less heat is liberated). It is advisable to carry out degassing as a matter of routine.

7. An alternative approach is to add a water-immiscible organic solvent, such as isobutanol, to the top of the gel. Less caution is obviously needed when adding this, although if using this approach, this step should be carried out in a fume cupboard, not in the open laboratory.
8. To save time some workers prefer to add the stacking gel solution directly and carefully to the top of the separating gel, i.e., the overlaying step (**step 7**) is omitted, the stacking gel solution itself providing the role of the overlaying solution.
9. Some workers include a small amount of bromophenol blue in this gel mix. This give a stacking gel that has a pale blue color, thus allowing the loading wells to be easily identified.
10. Even if the sample is loaded with too much vigor, such that it mixes extensively with the buffer in the well, this is not a problem, since the stacking gel system will still concentrate the sample.
11. When analyzing a sample for the first time, it is sensible to stop the run when the dye reaches the bottom of the gel, because there may be low mol-wt proteins that are running close to the dye, and these would be lost if electrophoresis was continued after the dye had run off the end of the gel. However, often one will find that the proteins being separated are only in the top two-thirds of the gel. In this case, in future runs, the dye would be run off the bottom of the gel, and electrophoresis carried out for a further 30 min to 1 h to allow proteins to separate across the full length of the gel thus increasing the separation of bands.
12. Normally, destain solution needs to be replaced at regular intervals since a simple equilibrium is quickly set up between the concentration of stain in the gel and destain solution, after which no further destaining takes place. To speed up this process and also save on destain solution, it is convenient to place some solid material in with the destain that will absorb the Coomassie dye as it elutes from the gel. We use a foam bung such as that used in culture flasks (ensure it is well wetted by expelling all air in the bung by squeezing it many times in the destain solution), although many other materials can be used (e.g., polystyrene packaging foam).
13. It is generally accepted that a very faint protein band detected by Coomassie brilliant blue, is equivalent to about 0.1 µg (100 ng) of protein. Such sensitivity is suitable for many people’s work. However if no protein bands are observed or greater staining is required, then silver staining (Chapter 48) can be further carried out on the gel.

14. Because the principle of this technique is the separation of proteins based on size differences, by running calibration proteins of known molecular weight on the same gel run as your unknown protein, the molecular weight of the unknown protein can be determined. For most proteins a plot of $\log_{10}$ molecular mass vs relative mobility provides a straight line graph, although one must be aware that for any given gel concentration this relationship is only linear over a limited range of molecular masses. As an approximate guide, using the system described here, the linear relationship is true over the following ranges: 15% acrylamide, 10,000–50,000; 10% acrylamide 15,000–70,000; 5% acrylamide 60,000–200,000. It should be stressed that this relationship only holds true for proteins that bind SDS in a constant weight ratio. This is true of many proteins but some proteins for example, highly basic proteins, may run differently than would be expected on the basis of their known molecular weight. In the case of the histones, which are highly basic proteins, they migrate more slowly than expected, presumably because of a reduced overall negative charge on the protein owing to their high proportion of positively-charged amino acids. Glycoproteins also tend to run anomalously presumably because the SDS only binds to the polypeptide part of the molecule.

    To determine the molecular weight of an unknown protein the relative mobilities (*Rf*) of the standard proteins are determined and a graph of log molecular weight vs *Rf* plotted.

    $$Rf = (\text{distance migrated by proteins/distance migrated by dye}) \qquad (3)$$

    Mixtures of standard mol-wt markers for use on SDS gels are available from a range of suppliers. The *Rf* of the unknown protein is then determined and the logMW (and hence molecular weight) determined from the graph. A more detailed description of protein mol-wt determination on SDS gels is described in **refs. *1*** and ***3***.

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22

Gradient SDS Polyacrylamide Gel Electrophoresis of Proteins

John M. Walker

1. Introduction

The preparation of fixed-concentration polyacrylamide gels has been described in Chapters 20 and 21. However, the use of polyacrylamide gels that have a gradient of increasing acrylamide concentration (and hence decreasing pore size) can sometimes have advantages over fixed-concentration acrylamide gels. During electrophoresis in gradient gels, proteins migrate until the decreasing pore size impedes further progress. Once the "pore limit" is reached, the protein banding pattern does not change appreciably with time, although migration does not cease completely ***(1)***. There are two main advantages of gradient gels over linear gels.

First, a much greater range of protein M_r values can be separated than on a fixed-percentage gel. In a complex mixture, very low-mol-wt proteins travel freely through the gel to begin with, and start to resolve when they reach the smaller pore size toward the lower part of the gel. Much larger proteins, on the other hand, can still enter the gel but start to separate immediately owing to the sieving effect of the gel. The second advantage of gradient gels is that proteins with very similar M_r values may be resolved, which otherwise cannot resolve in fixed percentage gels. As each protein moves through the gel, the pore size become smaller until the protein reaches its pore size limit. The pore size in the gel is now too small to allow passage of the protein, and the protein sample stacks up at this point as a sharp band. A similar-sized protein, but with slightly lower M_r, will be able to travel a little further through the gel before reaching its pore size limit, at which point it will form a sharp band. These two proteins, of slightly different M_r values, therefore separate as two, close, sharp bands.

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The usual limits of gradient gels are 3–30% acrylamide in linear or concave gradients. The choice of range will of course depend on the size of proteins being fractionated. The system described here is for a 5–20% linear gradient using SDS polyacrylamide gel electrophoresis. The theory of SDS polyacrylamide gel electrophoresis has been decribed in Chapter 21.

2. Materials

1. Stock acrylamide solution: 30% acrylamide, 0.8% *bis*-acrylamide. Dissolve 75 g of acrylamide and 2.0 g of *N,N′* -methylene *bis*-acrylamide in about 150 mL of water. Filter and make the volume to 250 mL. Store at 4°C. The solution is stable for months.
2. Buffers:
   a. 1.875 *M* Tris-HCl, pH 8.8.
   b. 0.6 *M* Tris-HCl, pH 6.8.
   Store at 4°C.
3. Ammonium persulfate solution (10% [w/v]). Make fresh as required.
4. SDS solution (10% [w/v]). Stable at room temperature. In cold conditions, the SDS can come out of solution, but may be redissolved by warming.
5. *N,N,N′ ,N′* -Tetramethylene diamine (TEMED).
6. Gradient forming apparatus (*see* **Fig. 1**). Reservoirs with dimensions of 2.5 cm id and 5.0 cm height are suitable. The two reservoirs of the gradient former should be linked by flexible tubing to allow them to be moved independently. This is necessary since although equal volumes are placed in each reservoir, the solutions differ in their densities and the relative positions of A and B have to be adjusted to balance the two solutions when the connecting clamp is opened (*see* **Note 3**).

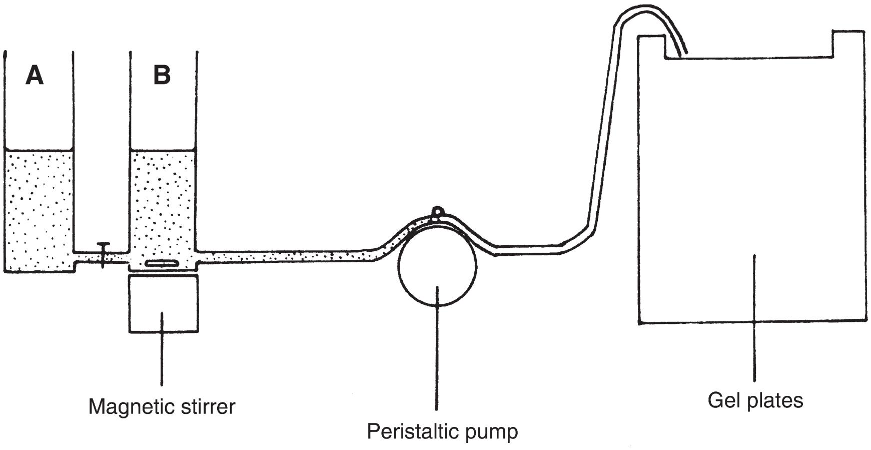

Fig. 1. Gradient forming apparatus.

3. Method

1. Prepare the following solutions:

	Solution A, mL	Solution B, mL
1.875 *M* Tris-HCl, pH 8.8	3.0	3.0
Water	9.3	0.6
Stock acrylamide, 30%	2.5	10.0
10% SDS	0.15	0.15
Ammonium persulfate (10%)	0.05	0.05
Sucrose	—	2.2 g (equivalent to 1.2 mL volume)

2. Degas each solution under vacuum for about 30 s and then, when you are ready to form the gradient, add TEMED (12 µL) to each solution.
3. Once the TEMED is added and mixed in, pour solutions A and B into the appropriate reservoirs (*see* **Fig. 1**.)
4. *With the stirrer stirring,* fractionally open the connection between A and B and adjust the relative heights of A and B such that there is no flow of liquid between the two reservoirs (easily seen because of the difference in densities). Do not worry if there is some mixing between reservoirs—this is inevitable.
5. When the levels are balanced, completely open the connection between A and B, turn the pump on, and fill the gel apparatus by running the gel solution down one edge of the gel slab. Surprisingly, very little mixing within the gradient occurs using this method. A pump speed of about 5 mL/min is suitable. If a pump is not available, the gradient may be run into the gel under gravity.
6. When the level of the gel reaches about 3 cm from the top of the gel slab, connect the pump to distilled water, reduce pump speed, and overlay the gel with 2–3 mm of water.
7. The gradient gel is now left to set for 30 min. Remember to rinse out the gradient former before the remaining gel solution sets in it.
8. When the separating gel has set, prepare a stacking gel by mixing the following:
   a. 1.0 mL 0.6 *M* Tris-HCl, pH 6.8;
   b. 1.35 mL Stock acrylamide;
   c. 7.5 mL Water;
   d. 0.1 mL 10% SDS;
   e. 0.05 mL Ammonium persulfate (10%).
9. Degas this mixture under vacuum for 30 s and then add TEMED (12 µL).
10. Pour off the water overlayering the gel and wash the gel surface with about 2 mL of stacking gel solution and then discard this solution.
11. The gel slab is now filled to the top of the plates with stacking gel solution and the well-forming comb placed in position (*see* Chapter 21).
12. When the stacking gel has set (~15 min), carefully remove the comb. The gel is now ready for running. The conditions of running and sample preparation are exactly as described for SDS gel electrophoresis in Chapter 21.

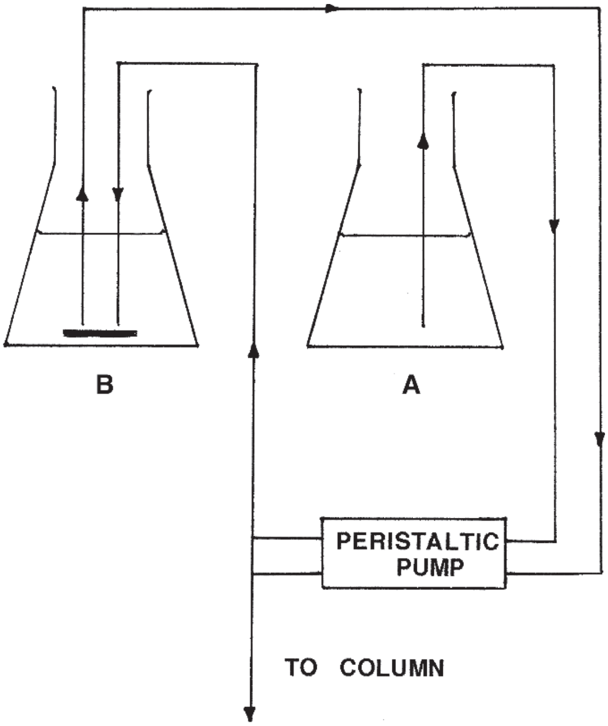

Fig. 2. Diagrammatic representation of a method for producing a gradient using a two-channel peristaltic pump. Reservoir B has the high percentage acrylamide concentration, reservoir A the lower.

4. Notes

1. The total volume of liquid in reservoirs A and B should be chosen such that it approximates to the volume available between the gel plates. However, allowance must be made for some liquid remaining in the reservoirs and tubing.
2. As well as a gradient in acrylamide concentration, a density gradient of sucrose (glycerol could also be used) is included to minimize mixing by convectional disturbances caused by heat evolved during polymerization. Some workers avoid this problem by also including a gradient of ammienium persulfate to ensure that polymerization occurs first at the top of the gel, progressing to the bottom. However, we have not found this to be necessary in our laboratory.

3. The production of a linear gradient has been described in this chapter. However, the same gradient mixed can be used to produce a concave (exponential) gradient. This concave gradient provides a very shallow gradient in the top half of the gel such that the percentage of acrylamide only varies from about 5–7% over the first half of the gel. The gradient then increases much more rapidly from 7–20% over the next half of the gel. The shallow part of the gradient allows high-mol-wt proteins of similar size to sufficiently resolve while at the same time still allowing lower mol-wt proteins to separate lower down the gradient. To produce a concave gradient, place 7.5 mL of solution B in reservoir B, then tightly stopper this reservoir with a rubber bung. Equalize the pressure in the chamber by briefly inserting a syringe needle through the bung. Now place 22.5 mL of solution A in reservoir A, open the connector between the two chambers, and commence pouring the gel. The volume of reservoir B will be seen to remain constant as liquid for reservoir A is drawn into this reservoir and diluted.
4. We have described the production of a linear gradient using a purpose built gradient mixer. However, it is not necessary to purchase this since the simple arrangement, shown in **Fig. 2** using just flasks or beakers, a stirrer, and a dual channel peristaltic pump, can just as easily be used.

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23

SDS-Polyacrylamide Gel Electrophoresis of Peptides

Ralph C. Judd

1. Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has proven to be among the most useful tools yet developed in the area of molecular biology. The discontinuous buffer system, first described by Laemmli ***(1),*** has made it possible to separate, visualize, and compare readily the component parts of complex mixtures of molecules (e.g., tissues, cells). SDS-PAGE separation of proteins and peptides makes it possible to quantify the amount of a particular protein/peptide in a sample, obtain fairly reliable molecular mass information, and, by combining SDS-PAGE with immunoelectroblotting, evaluate the antigenicity of proteins and peptides. SDS-PAGE is both a powerful separation system and a reliable preparative purification technique *(**2**;* and *see* Chapter 21*)*.

Parameters influencing the resolution of proteins or peptides separated by SDS-PAGE include the ratio of acrylamide to crosslinker (*bis*-acrylamide), the percentage of acrylamide/crosslinker used to form the stacking and separation gels, the pH of (and the components in) the stacking and separation buffers, and the method of sample preparation. Systems employing glycine in the running buffers (e.g., Laemmli ***[1]***, Dreyfuss et al. ***[3]***) can resolve proteins ranging in molecular mass from over 200,000 Daltons (200 kDa) down to about 3 kDa. Separation of proteins and peptides below 3 kDa necessitates slightly different procedures to obtain reliable molecular masses and to prevent band broadening. Further, the increased use of SDS-PAGE to purify proteins and peptides for N-terminal sequence analysis demands that glycine, which interferes significantly with automated sequence technology, be replaced with noninterfering buffer components.

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This chapter describes a modification of the tricine gel system of Schagger and von Jagow ***(4)*** by which peptides as small as 500 Daltons can be separated. This makes it possible to use SDS-PAGE peptide mapping (*see* Chapter 104); epitope mapping ***(5)***, and protein and peptide separation for N-terminal sequence analyses ***(6)*** when extremely small peptide fragments are to be studied. Since all forms of SDS-PAGE are denaturing, they are unsuitable for separation of proteins or peptides to be used in functional analyses (e.g., enzymes, receptors).

2. Materials

2.1. Equipment

1. SDS-PAGE gel apparatus.
2. Power pack.
3. Blotting apparatus.

2.2. Reagents

1. Separating/spacer gel acrylamide (1X crosslinker): 48 g acrylamide, 1.5 g *N,N′*-methylene-*bis*-acrylamide. Bring to 100 mL, and then filter through qualitative paper to remove cloudiness (*see* **Note 1**).
2. Separating gel acrylamide (2X crosslinker): 48 g acrylamide, 3 g *N,N′*-methylenebis-acrylamide. Bring to 100 mL, and then filter through qualitative paper to remove cloudiness (*see* **Note 2**).
3. Stacking gel acrylamide: 30 g acrylamide, 0.8 g *N,N′*-methylene-*bis*-acrylamide. Bring to 100 mL, and then filter through qualitative paper to remove cloudiness.
4. Separating/spacer gel buffer: 3 *M* Tris base, 0.3% sodium dodecyl sulfate (*see* **Note 3**). Bring to pH 8.9 with HCl.
5. Stacking gel buffer: 1 *M* Tris-HCl, pH 6.8.
6. Cathode (top) running buffer (10X stock): 1 *M* Tris base, 1 *M* tricine, 1% SDS (*see* **Note 3**). Dilute 1:10 immediately before use. Do not adjust pH; it will be about 8.25.
7. Anode (bottom) buffer (10X stock): 2 *M* Tris base. Bring to pH 8.9 with HCl. Dilute 1:10 immediately before use.
8. 0.2 *M* tetrasodium EDTA.
9. 10% ammonium persulfate (make fresh as required).
10. TEMED.
11. Glycerol.
12. Fixer/destainer: 25% isopropanol, 7% glacial acetic acid in dH_2O (v/v/v).
13. 1% Coomassie brilliant blue (CBB) (w/v) in fixer/destainer.
14. Sample solubilization buffer: 2 mL 10% SDS (w/v) in dH_2O, 1.0 mL glycerol, 0.625 mL 1 *M* Tris-HCl, pH 6.8, 6 mL dH_2O, bromphenol blue to color.
15. Dithiothreitol.
16. 2% Agarose.

17. Molecular-mass markers, e.g., low-mol-wt kit (Bio-Rad, Hercules, CA), or equivalent, and peptide molecular-mass markers (Pharmacia Inc., Piscataway, NJ), or equivalent.
18. PVDF (nylon) membranes.
19. Methanol.
20. Blotting transfer buffer: 20 m*M* phosphate buffer, pH 8.0: 94.7 mL 0.2 *M* Na_2HPO_4 stock, 5.3 mL 0.2 *M* NaH_2PO_4 stock in 900 mL H_2O.
21. Filter paper for blotting (Whatman No. 1), or equivalent.
22. Distilled water (dH_2O).

2.3. Gel Recipes

2.3.1. Separating Gel Recipe

Add reagents in order given (*see* **Note 4**): 6.7 mL water, 10 mL separating/spacer gel buffer, 10 mL separating/spacer gel acrylamide (1X or 2X crosslinker), 3.2 mL glycerol, 10 µL TEMED, 100 µL 10% ammonium persulfate.

2.3.2. Spacer Gel Recipe

Add reagents in order given (*see* **Note 4**): 6.9 mL water, 5.0 mL separating/spacer gel buffer, 3.0 mL separating/spacer gel acrylamide (1X crosslinker only), 5 µL TEMED, 50 µL 10% ammonium persulfate.

2.3.3. Stacking Gel Recipe

Add reagents in order given (*see* **Note 4**): 10.3 mL water, 1.9 mL stacking gel buffer, 2.5 mL stacking gel acrylamide, 150 µL EDTA, 7.5 µL TEMED, 150 µL 10% ammonium persulfate.

3. Methods

3.1. Sample Solubilization

1. Boil samples in sample solubilization buffer for 10–30 min. Solubilize sample at 1 mg/mL and run 1–2 µL/lane (1–2 µg/lane) (*see* **Note 5**). For sequence analysis, as much sample as is practical should be separated.

3.2. Gel Preparation/Electrophoresis

1. Assemble the gel apparatus (*see* **Note 6**). Make two marks on the front plate to identify top of separating gel and top of spacer gel (*see* **Note 7**). Assuming a well depth of 12 mm, the top of the separating gel should be 3.5 cm down from the top of the back plate, and the spacer gel should be 2 cm down from the top of the back plate, leaving a stacking gel of 8 mm (*see* **Note 8**).
2. Combine the reagents to make the separating gel, mix gently, and pipet the solution between the plates to lowest mark on the plate. Overlay the gel solution with

2 mL of dH_2O by gently running the dH_2O down the center of the inside of the front plate. Allow the gel to polymerize for about 20 min. When polymerized, the water–gel interface will be obvious.

3. Pour off the water, and dry between the plates with filter paper. Do not touch the surface of the separating gel with the paper. Combine the reagents to make the spacer gel, mix gently, and pipet the solution between the plates to second mark on the plate. Overlay the solution with 2 mL of dH_2O by gently running the dH_2O down the center of the inside of the front plate. Allow the gel to polymerize for about 20 min. When polymerized, the water–gel interface will be obvious.
4. Pour off the water, and dry between the plates with filter paper. Do not touch the surface of spacer gel with the paper. Combine the reagents to make the stacking gel and mix gently. Place the well-forming comb between the plates, leaving one end slightly higher than the other. Slowly add the stacking gel solution at the raised end (this allows air bubbles to be pushed up and out from under the comb teeth). When the solution reaches the top of the back plate, gently push the comb all the way down. Check to be sure that no air pockets are trapped beneath the comb. Allow the gel to polymerize for about 20 min.
5. When the stacking gel has polymerized, carefully remove the comb. Straighten any wells that might be crooked with a straightened metal paper clip. Remove the acrylamide at each edge to the depth of the wells. This helps prevent "smiling" of the samples at the edge of the gel. Seal the edges of the gel with 2% agarose.
6. Add freshly diluted cathode running buffer to the top chamber of the gel apparatus until it is 5–10 mm above the top of the gel. Squirt running buffer into each well with a Pasteur pipet to flush out any unpolymerized acrylamide. Check the lower chamber to ensure that no cathode running buffer is leaking from the top chamber, and then fill the bottom chamber with anode buffer. Remove any air bubbles from the under edge of the gel with a benttip Pasteur pipet. The gel is now ready for sample loading.
7. After loading the samples and the molecular-mass markers, connect leads from the power pack to the gel apparatus (the negative lead goes on the top, and the positive lead goes on the bottom). Gels can be run on constant current, constant voltage, or constant power settings. When using the constant current setting, run the gel at 50 mA. The voltage will be between 50 and 100 V at the beginning, and will slowly increase during the run. For a constant voltage setting, begin the electrophoresis at 50 mA. As the run progresses, the amperage will decrease, so adjust the amperage to 50 mA several times during the run or the electrophoresis will be very slow. If running on constant power, set between 5 and 7 W. Voltage and current will vary to maintain the wattage setting. Each system varies, so empirical information should be used to modify the electrophoresis conditions so that electrophoresis is completed in about 4 h (*see* **Note 9**).
8. When the dye front reaches the bottom of the gel, turn off the power, disassemble the gel apparatus, and place the gel in 200–300 mL of fixer/destainer. Gently shake for 16 h (*see* **Note 10**). Pour off spent fixer/destainer, and add CBB. Gently shake for 30 min. Destain the gel in several changes of fixer/destainer until the background is almost clear. Then place the gel in dH_2O, and gently mix until

the background is completely clear. The peptide bands will become a deep purple-blue. The gel can now be photographed or dried. To store the gel wet, soak the gel in 7% glacial acetic acid for 1 h, and seal in a plastic bag.

Figure 1 demonstrates the molecular mass range of separation of a 1X crosslinker tricine gel. Whole-cell (WC) lysates and 1X and 2X purified (*see* Chapter 104) 44 kDa proteins of *Neisseria gonorrhoeae*, Bio-Rad low-mol-wt markers (mw), and Pharmacia peptide markers (pep mw) were separated and stained with CBB. The top of the gel in this figure is at the spacer gel–separating gel interface. Proteins larger than about 100 kDa remained trapped at the spacer gel–separating gel interface, resulting in the bulging of the outside lanes. Smaller proteins all migrated into the gel, but many remained tightly bunched at the top of the separating gel. The effective separation range is below 40 kDa. Comparison of this figure with **Fig. 1** in Chapter 104, which shows gonococcal whole cells and the two mol-wt marker preparations separated in a standard 15% Laemmli gel ***(1)***, demonstrates the tremendous resolving power of low-mol-wt components by this tricine gel system.

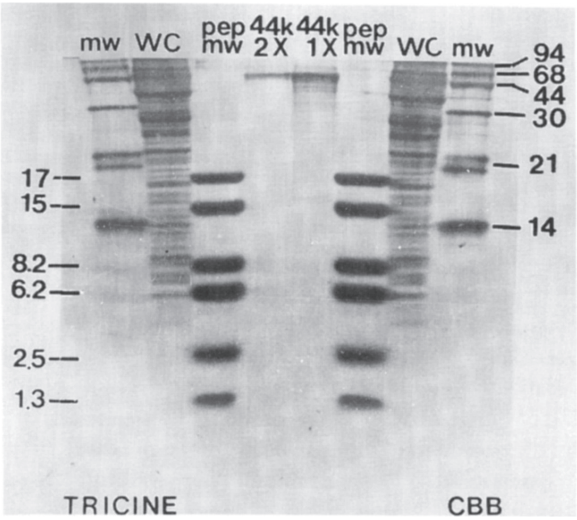

Fig. 1. WC Iysates and 1X and 2X purified 44 kDa protein of *Neisseria gonorrhoeae* (*see* Chapter 104, **Subheading 3.1.,**), Bio-Rad mw (1 µg of each protein), and Pharmacia pep mw (to which the 1.3-kDa protein kinase C substrate peptide [Sigma, St. Louis, MO] was added) (3 µg of each peptide), separated in a 1X crosslinker tricine gel, fixed, and stained with CBB. Molecular masses are given in thousands of daltons.

3.3. Blotting of Peptides

Separated peptides can be electroblotted to PVDF membranes for sequencing or immunological analyses (*see* **Note 11**).

1. Before the electrophoresis is complete, prepare enough of the 20 m*M* sodium phosphate transfer buffer, pH 8.0 (*see* **Note 12**) to fill the blotting chamber (usually 2–4 L). Degas about 1 L of transfer buffer for at least 15 min before use. Cut two sheets of filter paper to fit blotting apparatus, and cut a piece of PVDF membrane a little larger than the gel. Place the PVDF membrane in 10 mL of methanol until it is wet (this takes only a few seconds), and then place the membrane in 100 mL of degassed transfer buffer.
2. Following electrophoresis, remove the gel from the gel apparatus, and place it on blotting filter paper that is submersed in the degassed transfer buffer. Immediately overlay the exposed side of the gel with the wetted PVDF membrane, being sure to remove all air pockets between the gel and the membrane. Overlay the PVDF membrane with another piece of blotting filter paper, and place the gel "sandwich" into the blotting chamber using the appropriate spacers and holders.
3. Connect the power pack electrodes to the blotting chamber (the positive electrode goes on the side of the gel having the PVDF membrane). Electrophorese for 16 h at 25 V, 0.8 A. Each system varies, so settings may be somewhat different than those described here.
4. Following electroblotting, disconnect the power, disassemble the blotting chamber, and remove the PVDF membrane from the gel (*see* **Note 13**). The PVDF membrane can be processed for immunological analyses or placed in CBB in fixer/destainer to stain the transferred peptides. Remove excess stain by shaking the membrane in several changes of fixer/destainer until background is white. Peptide bands can be excised, rinsed in dH_2O, dried, and subjected to N-terminal sequencing.

3.4. Modifications for Peptide Sequencing

Peptides to be used in N-terminal sequence analyses must be protected from oxidation, which can block the N-terminus. Several simple precautions can help prevent this common problem.

1. Prepare the separation and spacer gel the day before electrophoresing the peptides. After pouring, overlay the spacer gel with several milliliters of dH_2O, and allow the gel to stand overnight at room temperature (*see* **Note 14**).
2. On the next day, pour off the water, and dry between the plates with filter paper. Do not touch the gel with the filter paper. Prepare the stacking gel, but use half the amount of 10% ammonium persulfate (*see* **Note 15**). Pipet the stacking gel solution between the plates as described above, and allow the stacking gel to polymerize for at least 1 h. Add running buffers as described above, adding 1–2 mg of dithiothreitol to both the upper and lower chambers to scavenge any oxidizers from the buffers and gel.
3. Pre-electrophorese the gel for 15 min. then turn off the power, and load the samples and molecular-mass markers.

4. Run the gel as described above.
5. Blot the peptides to a PVDF membrane as described above, but add 1–2 mg of dithiothreitol to the blot transfer buffer.
6. Fix and stain as above, again adding 1–2 mg of dithiothreitol to the fixer/destainer, CBB, and dH_2O used to rinse the peptide-containing PVDF membrane.

Acknowledgments

The author thanks Joan Strange for her assistance in developing this system, Pam Gannon for her assistance, and the Public Health Service, NIH, NIAID (grants RO1 AI21236) and UM Research Grant Program for their continued support.

4. Notes

1. Working range of separation about 40 kDa down to about 1 kDa.
2. Working range of separation about 20 kDa down to less than 500 Dalton.
3. Use electrophoresis grade SDS. If peptide bands remain diffuse, try SDS from BDH (Poole, Dorset, UK).
4. Degassing of gel reagents is **not** necessary.
5. Coomassie staining can generally visualize a band of 0.5 µg. This may vary considerably based on the properties of the particular peptide (some peptides stain poorly with Coomassie). Some peptides do not bind SDS well and may never migrate exactly right when compared to mol-wt markers. Fortunately, these situations are rare.
6. Protocols are designed for a standard 13 cm × 11 cm × 1.5 mm slab gel. Dimensions and reagent volumes can be proportionally adjusted to accommodate other gel dimensions.
7. Permanent marks with a diamond pencil can be made on the back of the back plate if the plate is dedicated to this gel system.
8. The depth of the spacer gel can be varied from 1 to 2 cm. Trial and error is the only way to determine the appropriate dimension for each system.
9. It is wise to feel the front plate several times during the electrophoresis to check for over-heating. The plate will become pleasantly warm as the run progresses. If it becomes too warm, the plates might break, so turn down the power!
10. Standard-sized gels can be fixed in as little as 4 h with shaking.
11. It is best to blot peptides to PVDF membranes rather than nitrocellulose membranes, since small peptides tend to pass through nitrocellulose without binding. Moreover, peptides immobilized on PVDF membranes can be directly sequenced in automated instrumentation equipped with a “blot cartridge” ***(6)***.
12. The pH of the transfer buffer can be varied from 5.7 to 8.0 if transfer is inefficient at pH 8.0 ***(7)***.
13. Wear disposable gloves when handling membranes.

14. Do not refrigerate the gel. It will contract and pull away from the plates, resulting in leaks and poor resolution.
15. Do not pour the stacking gel the day before electrophoresis. It will shrink, allowing the samples to leak from the wells.

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24

Separation of Proteins by Blue Native Electrophoresis (BN-PAGE)

Elke A. Dian, Joachim Rassow, and Christian Motz

1. Introduction

Proteins often work together in protein complexes. For the analysis of the function as well as the structure of proteins it is often of interest to analyze the assembly of such complexes. While protein complexes normally dissociate into their subunits when analyzed by SDS-PAGE **(Fig. 1B)**, nondenaturing electrophoresis (so called native electrophoresis) methods leave the complexes intact. Blue native polyacrylamide gel electrophoresis (BN-PAGE, **Fig. 1C**), a method developed by Schägger and Jagow ***(1)***, has proven to be a valuable tool to analyze the respiratory chain complexes of different organisms ***(2,3)*** to investigate the complexes of the import machinery of mitochondria ***(4–6)*** or chloroplasts ***(7)***, or to analyze the proteome of isolated organelles or whole cellular lysates ***(8,9)***.

Compared to other methods, BN-PAGE offers some major advantages: (1) It allows the determination of the molecular weight for both positively and negatively charged proteins as well as for membrane proteins. (2) In contrast to gel filtration, BN-PAGE offers the possibility of testing many samples on the same gel (i.e., under identical conditions). (3) Small samples (electrophoretic amounts down to 5 µg) are possible. (4) The electrophoresis can be performed using standard equipment for SDS-PAGE.

With other native electrophoresis methods, the mobility of the proteins is mainly determined by their intrinsic charge but also to some extend by their size. Therefore neither the pI nor the molecular weight of the proteins can be exactly determined. The BN-PAGE overcomes this difficulty by binding of the anionic dye Coomassie Blue G-250 and so inducing a charge shift to the proteins. Thus the electrophoretic mobility depends only on the molecular weight.

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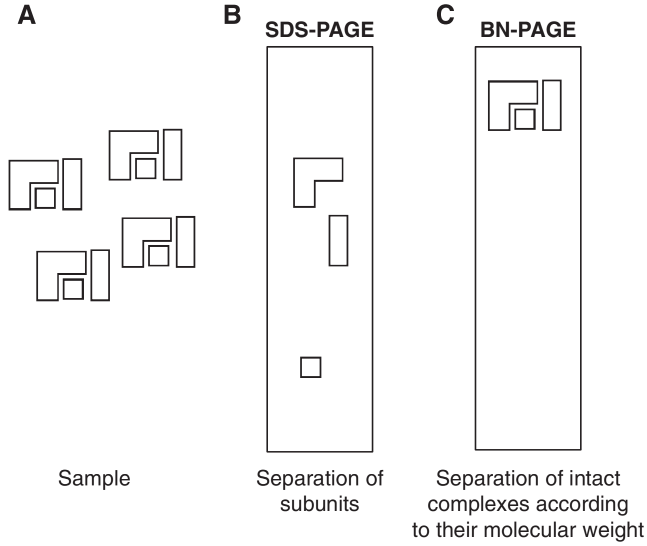
A
B
SDS-PAGE
C
BN-PAGE
Sample
Separation of subunits
Separation of intact complexes according to their molecular weight

Fig. 1. Principle of the separation with different electrophoretic techniques. **(A)** The proteins to be separated. **(B)** SDS-PAGE: the subunits are separated according to their size. **(C)** The intact protein complexes are separated by size.

Schägger et al. *(10)* showed that the BN-PAGE allows the analysis of molecular masses of most soluble as well as most membrane proteins. Only a few proteins that do not bind the Coomassie dye and have no or almost no negative charge at pH 7.5 showed a significantly different behavior. As a side effect, the negatively charged dye also prevents aggregation of the proteins.

While in SDS-PAGE the SDS / protein ratio and thus the charge / mass ratio is constant for all proteins, the ratio of bound dye / protein shows some variation in BN-PAGE. Especially integral membrane proteins show an increase in their apparent molecular weights compared to soluble proteins due to differences in the affinity for detergent or dye molecules. Heuberger et al. *(11)* showed for different membrane proteins that bound detergents were replaced by dye molecules during electrophoresis. The apparent molecular weight was increased by the factor 1.8 compared to the molecular weight of soluble proteins that served as marker proteins.

2. Materials

Prepare all solutions in distilled H_2O unless otherwise stated.

1. Electrophoresis chamber: the gel has to be cooled during electrophoresis. Although running the gel in the cold room in a standard electrophoresis chamber may be

Table 1
Composition of Separating and Stacking Gels

	Separating gel: acrylamide concentration					
Components	*4 %*	*6 %*	*13 %*	*16 %*	*20 %*	Stacking gel
AB-mix	0.75 mL	1.15 mL	2.35 mL	3 mL	3.75 mL	0.3 mL
Gel buffer	3 mL	3 mL	3 mL	3 mL	3 mL	1.25 mL
Glycerol	-	-	1 mL	1 mL	1 mL	-
H_2O	Fill to 9 mL					2.2 mL
APS	38 µL	38 µL	30 µL	30 µL	30 µL	30 µL
TEMED	3.8 µL	3.8 µL	3 µL	3 µL	3 µL	3 µL

sufficient, best cooling is achieved using a system with cooling in a water bath, such as the Hoefer SE600 system (*see* **Note 1**).

2. A gradient mixer of the appropriate volume for a separating gel (*see* **Table 1** and **Note 7**).
3. Cathode buffer A: 50 m*M* Tricine, 15 m*M* Bis-Tris, 0.02 % coomassie brilliant blue G-250, pH 7.0 at 4°C, do not adjust the pH (*see* **Notes 1** and **2**).
4. Cathode buffer B: 50 m*M* Tricine, 15 m*M* Bis-Tris, pH 7.0 at 4°C (*see* **Note 1**).
5. Anode buffer: 50 m*M* Bis-Tris, adjust pH 7.0 with HCl (*see* **Note 1**).
6. Gel Buffer (3x): 1,5 *M* ε-aminocaproic acid, 150 m*M* Bis-Tris (*see* **Note 3**).
7. AB-Mix: 49.5 % acrylamide, 1,5 % bisacrylamide.
8. APS: 10 % (w/v) ammoniumpersulfate in H_2O.
9. TEMED: N,N,N’,N’-tetrametylethylenediamine.
10. Lysis buffer: 20 m*M* Tris-HCl, 0.1 m*M* EDTA, 500 m*M* ε-aminocaproic acid, 10 % glycerol, pH 7.0 (HCl). Complete with the desired detergent (*see* **Notes 3 – 6**) and add PMSF to 1 m*M* just before use.
11. PMSF: phenylmethylsulfonylfluoride 200 m*M* in 2-propanol.
12. Sample buffer: 500 m*M* ε-aminocaproic acid, 100 m*M* Bis-Tris, 5 % Serva Blue G, pH 7.0 (HCl) (*see* **Note 2**).
13. 100 % methanol.
14. Blot buffer: 20 m*M* Tris, 150 m*M* glycine, 0.02 % SDS, 20 % methanol, do not adjust the pH.

3. Methods

3.1. General Experiment

3.1.1. Preparing the Gel

1. Prepare the gel assembly according to the manufacturers instructions,
2. Mix the separating gel solutions (**Table 1,** *see* **Notes 7** and **8**) without APS and fill them into a gradient mixer, filling the higher concentration in the exit chamber. Add the APS and TEMED, then pour the gel. Overlay with 2-propanol or 1-butanol.
3. After the separating gel has polymerized, discard the alcohol, then mix and pour the stacking gel **(Table 1).** Place a comb in the gel to form the wells.

3.1.2. Sample Preparation

The sample preparation depends on whether the proteins to be analyzed are soluble, or membrane proteins. For first experiments start with or twice the amount you would use for a normal SDS-PAGE (*see* **Note 9**). Soluble proteins can be used without further preparation by dilution with lysis buffer and addition of 10 % (v/v) sample buffer and proceed with the electrophoresis. Already solubilized membrane proteins can be treated in a similar manner if the detergent does not interfere with the electrophoresis (*see* **Note 10**).

Ideally, the proteins are precipitated or in membranes. Such samples are treated in the following way: Prior to the electrophoresis these samples are lysed in a buffer containing detergent. The choice of detergent is crucial because too harsh conditions will dissociate the complexes whereas too mild conditions may not be sufficient to solubilize the proteins (*see* **Fig. 2B** and **Notes 4–6**).

1. Reisolate the membranes (vesicles or organelles) or the protein precipitate by centrifugation. Discard the supernatant.
2. Resuspend the samples in 70 µL lysis buffer (i.e. 1 µL per µg protein) containing the detergent and 1 m*M* PMSF.

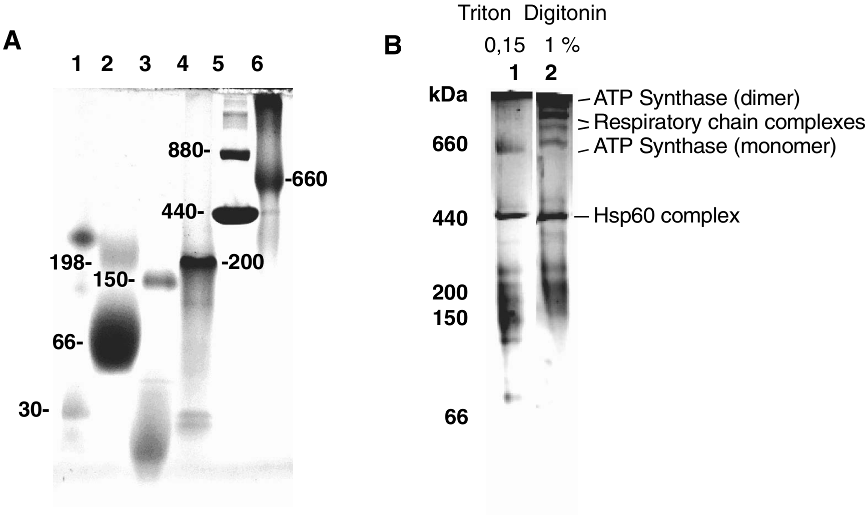

Fig. 2. Separation of protein complexes by BN-PAGE (4–16 % acrylamide gradient), staining with Coomassie. **(A)** Marker proteins: lane 1: carbonic anhydrase (30 kDa), 2: BSA (66 kDa), 3: alcohol dehydrogenase (150 kDa), 4: β-amylase (200 kDa), 5: apoferritin (440 kDa), 6: thyroglobulin (660 kDa). Note that some of the markers give more than one band; therefore it is not recommended to mix all the markers in one lane. **(B)** Separation of proteins from isolated yeast mitochondria in BN-PAGE (4–16 % gradient): lane 1: lysis of mitochondria with Triton X-100, lane 2: lysis with digitonin. Note that the large complexes of the respiratory chain are visible only when the mitochondria were lysed in digitonin. The ATP synthase dimer is only visible in digitonin.

3. Incubate 20 min on ice (*see* **Note 6**).
4. Centrifuge 20 min at 20000 g (minimum, use an ultracentrifuge if available) to pellet insoluble aggregates.
5. Discard the pellet (or keep it for lysis control using SDS-PAGE).
6. Add 7 µL of sample buffer (i.e. 10 % of the sample volume) to the supernatant.

3.1.3. Electrophoresis

1. Apply the samples to the gel.
2. Apply the markers solubilized in sample buffer (*see* **Note 11**).
3. Overlay the samples with cathode buffer A, assemble the precooled electrophoresis unit and fill the cathode buffer A in the upper buffer chamber.
4. Start the electrophoresis at 200 V until the blue front reaches the separating gel, then set the voltage to 500 V with the current limited to 15 mA per gel (*see* **Note 12**).
5. Removing excess dye can be achieved by changing the cathode buffer A to cathode buffer B after about one third of the run. This is recommended when blotting the gel but can be omitted if the gel will be stained after electrophoresis.

3.1.4. Staining

The Serva Blue G will not stain all proteins, so it will be necessary to stain the gel according to your standard procedures, e.g. staining with Coomassie Blue R-250 or silver staining.

3.1.5. Western Blotting

For western blotting it is best to use polyvinylidene (PVDF) membranes, which will allow removing the dye using organic solvents. Note that, unlike nitrocellulose, PVDF needs an extra preparation.

1. Prepare the PVDF membrane by immersing for a few seconds in 100 % methanol, until the membrane is translucent. Wash with water for another five min.
2. Incubate the membrane a few minutes in blot buffer. The membrane submerges when it is equilibrated.
3. Equilibrate the gel for a few seconds in transfer buffer.
4. Perform the western blotting according to your standard procedures.
5. After blotting, remove the blue dye by washing the membrane with methanol, then rinse with water.
6. The membrane can be stained or used for antibody detection following your standard procedures. Note that if the membrane once gets dry, you will have to repeat **step 1** before going on.

3.2. Two-Dimensional Gels

1. After running the native gel, disassemble the glass plates and remove the stacking gel.
2. Cut out the lanes of interest.
3. Place the lane on a glass plate at the usual position of the stacking gel. Leave some space at the side for a marker or a control (*see* **Fig. 3A** and **Note 13**).

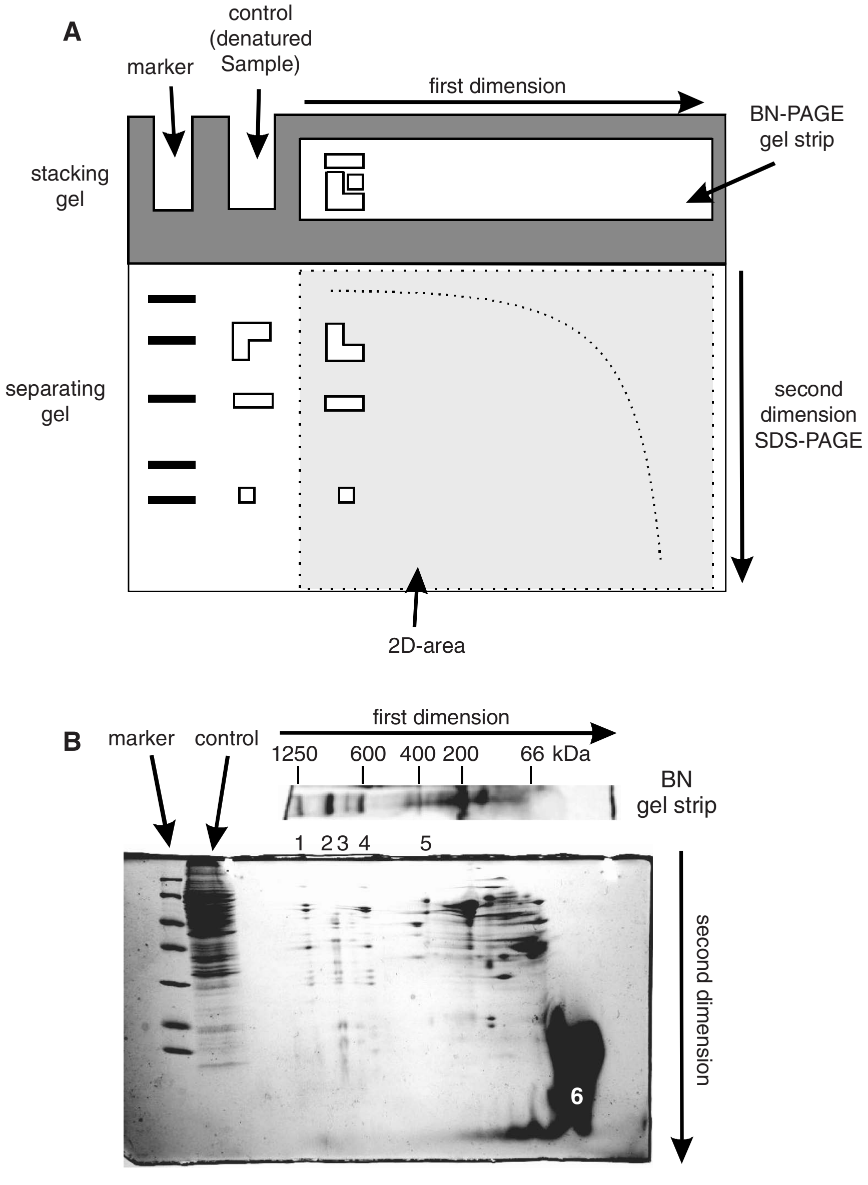
A
marker
control (denatured Sample)
first dimension
BN-PAGE gel strip
stacking gel
separating gel
second dimension SDS-PAGE
2D-area
B
marker
control
first dimension
1250
600
400
200
66 kDa
BN gel strip
1
2
3
4
5
6
second dimension

Fig. 3. Two-dimensional gel. **(A)** Principle; in the first dimension (BN-PAGE, left to right), the protein complexes are separated, in the second dimension (SDS-PAGE, top-down), the subunits and monomers are separated. The subunits of one complex can be found one below the other. Monomers will be found on the dotted hyperbolic line. **(B)** Example: mitochondria (70 µg protein) lysed in digitonin: 1. ATP synthase (dimer), 2, 3: respiratory chain complexes, 4: ATP Synthase (monomer), 5: Hsp60 complex, 6: excess Coomassie dye

4. Assemble the glass plates for a standard SDS gel. The excised gel strip will be held in its position by friction.
5. Pour the separating gel. Leave about 0.5–1 cm below the gel strip. Overlay with 2-propanol or 1-butanol (according to your standard procedures).
6. After polymerization, decant the propanol and pour the stacking gel, avoiding air bubbles under the gel strip. This can be achieved by tilting the gel assembly and slowly straighten it while pouring the gel. Place a small comb for one or two wells for marker and control (*see* **Note 13**).
7. Place the gel into the electrophoresis apparatus. Apply the marker. If you need reducing conditions, overlay the blue native strip with reducing sample buffer.
8. Perform the electrophoresis according to your standard protocols. The blue dye in the native gel strip will form a thin line. After the line has reached the separating gel, remove the gel strip with a thin spatula. This will avoid an uneven run.
9. The gel can be stained, blotted, and so on, according to your standard procedures.

3.3. Analysis of Organelles or Cell Lysates

Blue Native electrophoresis offers the posibility to identify protein complexes in proteomic approaches. Camacho-Carvajal et al. *(9)* subjected whole cellular lysates to BN-PAGE with subsequent SDS-PAGE and identified various protein complexes using immunoblotting. Werhahn and Braun *(8)* used an elegant three-dimensional electrophoresis to identify the proteome of the mitochondrial complexes: in a first dimension they subjected mitochondria to BN-PAGE. The resulting bands of the protein complexes were cut out and each was subjected to first dimension isoelectric focusing and subsequent second dimension SDS-PAGE, resulting in a set of "classic" two dimensional gels, each representing the proteome of one complex of mitochondrial proteins.

3.4. Analysis of Isolated Protein Complexes

Figure 4A shows an assembly experiment with F_1 ATPase from *Escherichia coli* monitored by Blue Native electrophoresis: isolated α, β, and γ subunits were mixed in a stoichometric ratio without (lane 2) and with (lane 3) MgATP and then analyzed by BN-PAGE. Lane 1 shows intact ATPase as a control. Whereas in the absence of MgATP, only a mixture of the subunits can be detected (lane 2), in the presence of MgATP, a larger complex is detected (lane 3, arrow) which corresponds to $\alpha_3\beta_3\gamma$.

Arnold et al. *(12)* analyzed isolated ATP synthase from yeast with Blue Native electrophoresis. They could show the existence of two different complexes: one with the molecular weight of the ATP synthase, one with the double weight. Using a subsequent second dimension SDS-PAGE, they could identify both complexes as ATP synthase and further identify some proteins which are only present in the dimer.

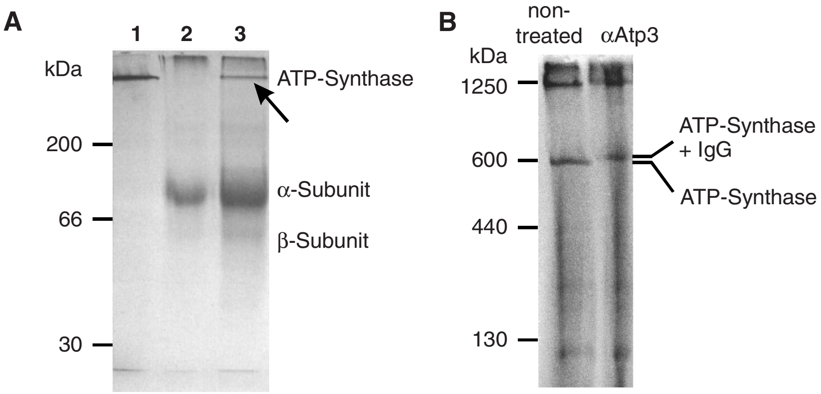

Fig. 4. Examples for applications of BN-PAGE: A: BN-PAGE of an assembly experiment with F_1 ATPase from *Escherichia coli*: 1: F1-ATPase, 2: isolated α, β und γ subunits (3:3:1) without MgATP, 3: isolated α, β und γ subunits (3:3:1) with MgATP. The arrow indicates the presence of a reconstituted $\alpha_3\beta_3\gamma$ complex. B: Autoradiogram of a BN-PAGE with mitochondria after the import of radiolabeled γ-subunit (Atp3) and lysis with digitonin. The right sample is treated with antiserum against Atp3. Due to binding of the IgG molecules to the ATP synthase complex, the corresponding bands are shifted from 600 kDa to 750 kDa.

3.5. Using an Antibody Shift to Determine Interaction Partners

Antibody binding is not affected by BN-PAGE and can thus be detected by a molecular mass shift of about 150 kDa (the molecular weight of a IgG molecule). Wiedemann et al. ***(13)*** used this method to identify components of the assembly intermediates of the TOM complex. This method can be used as an alternative to coimmune precipitation. Dissolve the samples in lysis buffer containing 1 % digitonin and add 1–5 µL polyclonal antiserum against potential interaction partners. Use a sample with the same amount of preimmune serum as control. Incubate 20 min on ice. Centrifuge, then run the blue native gel as described in **Subheading 3.1**.

Detect your protein of interest by western blotting or phosphor imager analysis. Protein bands showing an increase of the molecular weight by 150 or 300 kDa in comparison with the control indicate the binding of one or two IgG molecules and thus the presence of the protein the antibody was raised against in the protein complex of interest **(Fig. 4B)**.

4. Notes

1. The Hoefer SE600 system needs 400 mL of cathode buffer and 4 L of anode buffer, both can be reused up to five times
2. Coomassie Brilliant Blue R-250 cannot be used.

3. Schägger and Jagow ***(1)*** use a concentration of 500 m*M* ε-aminocaproic acid (EACA) in the gel and 750 m*M* in the lysis buffer. The concentration, however, can be varied in a wide range (100 m*M* to 1 *M*).
4. The choice of detergent is a critical step, as shown in **Fig. 2B**. For yeast mitochondria, 1 % digitonin will give a good lysis while keeping most protein complexes together. Also Triton X-100 in a range from 0,2 to 2 % may give good results. Any other detergent may be used; it should be selected to extract the proteins and preserve the protein complexes (*see* **Note 6**).
5. For determination of the protein pattern of the monomers, 1 % SDS or 8 *M* urea can be used.
6. The appropriate lysis conditions (lysis time, lysis buffer, detergent) should be determined in a pilot experiment: lyse the samples under different conditions, centrifuge at 20000 g (better: 100000 g) and analyze the pellet and supernatant on a SDS-gel for the protein of interest. Select conditions that keep the protein in the supernatant.
7. A linear gradient range from 4–16 % will give a good resolution from 1000 to 100 kDa, for other molecular weight ranges, gradients between 4 and 20% can be used. For separation of proteins in the range from 50 to 100 kDa, a homogenous gel with 10% acrylamide has proven suitable ***(14)***.
8. The volume of 18 mL for the separating gel is convenient for a gel with the dimensions 18 × 16 cm and 1 mm thickness, as purchased with the Hoefer SE600. For other gels, the appropriate volume has to be determined. The use of spacers with more than 1 mm thickness may result in broadening of the protein bands.
9. Protein amounts of 50 to 100 µg per sample for a heterogeneous mixture of proteins and 5 to 30 µg for purified protein are usually satisfactory detectable. For first experiments use twice the protein as you would for a normal experiment analyzed by SDS-PAGE. A high background or smearing on the gel, however, can be reduced by reducing the sample size.
10. It may be possible that some substances in the protein solution may interfere with the electrophoresis, so a precipitation or dialysis with lysis buffer may be necessary.
11. Molecular weight markers for gel filtration calibration (e.g. Sigma MW-GF-1000, *see* **Fig. 2A**) can be used as markers. Some of the markers may give more than one band, so it is best to run each marker in a separate lane first to see which may be mixed without confusing the bands (e.g., BSA and Apoferritin).
12. The Gel will run approximately five hours. Set the Voltage to 50 V if you want to run the gel overnight.
13. For analyzing protein mixtures with two-dimensional gels, it is good to apply an aliquot from the same experiment in Laemmli buffer (SDS-Buffer) as control on the side of the gel. A control of a SDS denatured sample that contains the protein of interest will make it easier to find the corresponding spots on the 2-D Gel. It will also facilitate troubleshooting.

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25

Separation of Proteins by Gel Electrophoresis in the Tris-Taurine-HCl System

**Sylvie Luche, Mireille Chevallet, Cécile Lelong,
and Thierry Rabilloud**

1. Introduction

SDS-PAGE (i.e., zone electrophoresis of proteins in the presence of SDS) is perhaps one of the most widely used protein separation tool. This is due to its relatively ease of setup, its robustness, in the sense that near all proteins are amenable to this type of separation, its compatibility with further protein analysis techniques (e.g., by blotting or by mass spectrometry), and its good resolution. The resolution of zone electrophoresis has been vastly improved by the use of discontinuous systems ***(1)***, so that almost only discontinuous systems are used nowadays, and the popular Laemmli system is just one of those ***(2)***.

However, there is no universal solution in protein separation, so that there is no single gel that is able to exhibit optimal protein separation for all studies. Quite often, the resolution must be adjusted to the experimental needs. These can vary from a rather low resolution over a wide range of molecular masses to a high resolution in a limited molecular mass range.

Most often, resolution is adjusted to the experimental needs by playing with the acrylamide concentration. By changing the pore size in the resulting gel, this results in changed sieving properties and thus to changed retardation of proteins in the gels. While very efficient, this way of adjusting resolution is not without practical drawbacks. Highly concentrated gels (15%acrylamide and over) are rather brittle, while low concentration gels (7.5% and lower) are very soft and tend to break. Moreover, highly concentrated gels pose often technical problems in the subsequent steps (e.g., in the mobilization of proteins for blotting or in the efficient penetration of trypsin in proteomics experiments).

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However, there is another way to adjust resolution in SDS-PAGE, which is to play with the electrophoresis buffer system. In discontinuous electrophoresis, the migration is stopped when the ion boundary (i.e., the zone materializing the migration of the fastest ions) has reached the bottom of the gels. This means in turn that the resolution of the proteins is the ratio between their mobility in the gel and the mobility of this ion boundary, and this gives another mechanism to tune protein resolution.

For example, at equal gel concentration, if the ion boundary mobility is slow, this means that all the small, fast-moving proteins will travel with the ion boundary with no resolution. Meanwhile, as the total electrophoretic process is slower and thus takes longer, the slow moving proteins will be given more time to travel in the gel and thus achieve greater resolution if the gel has adequate sieving properties. The overall result is an increased resolution of the high molecular weight proteins and a decreased resolution of low molecular weight proteins.

Conversely, if the ion boundary is fast, even the fast-moving, low molecular weight proteins will not be able to travel as fast as the ion boundary, mainly because of gel sieving. As a result, separation will show. However, as in this case the migration time is short, high molecular weight proteins travel minimally in the gel and are therefore poorly resolved. Of course, both acrylamide concentration and ion boundary can be adjusted independently to provide the optimal resolution.

While the Tris-glycine system originally described by Ornstein and Davis ***(1,3)*** is still by far the most popular, it is not a versatile system in resolution adjustment, and this illustrates how the adjustment of ion boundary speed works. Ion boundary mobilities can be calculated by using the system described by Jovin ***(4)***, but more intuitive rules can be used to understand what happens.

Ion boundary mobility is just a subcase of isotachophoresis, and a consequence is that the boundary speed is the speed of the slowest-moving ion. Quite often, the low mobility of this trailing ion is tuned by choosing a zwitterionic compound with basic and acidic pKs, so that changing the pH will change the mobility of the ion.

Going back to the example of a glycine-based anionic system, the basic pK of glycine is 9.7. This means at an operating pH of 9.7, the speed of the glycine ion wil be one half of its absolute mobility, as half of the molecules are charged. At 8.7, it will be one tenth of the absolute mobility, and 90% of the absolute mobility at 10.7.

While this is not a problem per se, these values pose practical problems. For example, at pH 10.7, the acrylamide gel will start to hydrolyze, giving rise to electroosmosis and mechanical problems. Furthermore, while the operating pH is not the gel pH (it is higher in anionic systems), the operating pH is dictated by the pH of the gel, which itself is governed by the other, cationic buffering component—Tris in the case of Tris-glycine gels. As the pK of Tris is close to 8.1, this means that the buffering capacity becomes low at pH 9 and this alters the robustness of the system. It can be argued that other buffering components than Tris can be used. While this is in principle true, this buffer is the gel polymerization buffer. This means that this buffering component must not interfere with the acrylamide polymerization. As a result, choice of buffers that can be used is very limited. For example, only ammediol has been used to operate gels at high pH and thus allow for the resolution of low-molecular weight proteins in glycine systems ***(5)***.

At the other end of the spectrum, decreasing the pH below the classical 8.8 value of the Ornstein-Davis system enables better resolution in the high molecular mass range ***(6,7)***. Thus, alternate systems have been proposed for the resolution of low-molecular weight proteins. As a result of the high performance of Tris as a polymerization buffer, these systems have used zwitterionic compounds which a much lower pK than glycine, for example Tricine ***(8)***, Bicine ***(9)***, or even MES ***(10)***.

In addition, the use of high pH for gels electrophoresis is not without drawbacks. High pH gels have limited shelf-life because of slow hydrolysis of the acrylamide. Moreover, the reactivity of amino acids toward residual acrylamide monomer is increased at high pH, leading to modification of the side chains ***(11)***.

It has been therefore proposed to use a neutral pH system using Tricine as a trailing ion ***(12)***. However, here again, the required pH of 7 is far from the pK of Tris, leading to robustness problems.

The conclusion of these statements is that Tricine, pK 8, is well adapted to high velocity buffers while not adapted to low velocity buffers, while the reverse situation is encountered with glycine and its pK 9.7. We therefore reasoned that a system using a trailing ion of pK close to 8.8 should be adaptable to both high and low velocity buffers, while operating at moderately basic values and close to the pK of Tris—and therefore at maximal buffer capacity.

Additional constraints in the possible trailing ions, such as cost, or the presence of a primary amine to ensure compatibility with all types of silver staining, further limited the choice, and we selected taurine as a trailing ion ***(13)***. Once this choice was made, we could devise a double gel system (stacking-separating gel) to further optimize the resolution.

2. Materials

2.1. Stock Solutions (All Stored at Room Temperature)

1. Stock acrylamide solution: 30% acrylamide, 0.8% bis-acrylamide.
2. Buffers (*see* **Note 1**):
   a. Sample stock buffer: 120 g/l Tris, 0.8 M HCl.

   b. Stacking buffer: 90 g/l Tris, 0.6 M HCl.
   c. Separating buffer, low speed: 110 g/l Tris, 0.6 M HCl.
   d. Separating buffer, medium speed: 130 g/l Tris, 0.6 M HCl.
   e. Separating buffer, high speed: 150 g/l Tris, 0.6 M HCl.
3. 20% SDS.
4. 10% ammonium persulfate, made fresh every week.
5. Cathode buffer: 6.g/l Tris, 1.g/l SDS, 25.g/l taurine (*see* **Note 2**).
6. Anode buffer: 6 g/l Tris, 1 g/l SDS, 30 g/l glycine (*see* **Note 2**).
7. TEMED.
8. 60% glycerol.
9. Thioglycerol (*see* **Note 3**).
10. Saturated bromophenol blue in water.
11. Solid urea.
12. 2-butanol, saturated with an equal volume of water, and kept as a two-phase system.

2.2. Gel Recipes and Sample Buffers

1. Concentrated sample buffer for 1D electrophoresis: Mix 250 μl of stacking buffer, 250 μl of 20% SDS, 350μl of glycerol, 100 μl of thioglycerol, and 5 μl of saturated bromophenol blue. Add water up to 1 ml.
2. Gel equilibration buffer for 2D electrophoresis: Mix 25 ml of stacking buffer, 25 ml of 20% SDS and 100 ml of glycerol. Add 72 g of urea. This makes directly 200 ml of gel equlibration buffer.
3. Stacking gel: Mix 1 ml of stacking gel buffer, 1 ml of stock acrylamide solution, and 4 ml of water. Add 6 μl of TEMED, and finally 60 μl of 10% ammonium persulfate.
4. Separating gel (*see* **Note 4**): Mix 10 ml of the selected separating gel buffer, 20 ml of stock acrylamide solution and 30 m of water. Add 20 μl of TEMED and finally 400 μl of 10% ammonium persulfate.
5. Agarose gel for two-dimensional electrophoresis: To 1 g of agarose, add 12.5 ml of sample stock buffer, 2 ml of 20% SDS, 2 ml of saturated bromophenol blue solution. Add water to 100 ml, and dissolve the agarose by boiling. Store in 10 ml aliquots and let the gel set.

3. Methods

3.1. Gel Casting for One-Dimensional Gel Electrophoresis

3.1.1. Sample Solubilization

Mix an equal volume of sample with a volume of concentrated sample buffer. Incubate at 100°C (boiling water bath) for 5 min. Let cool at room temperature

3.1.2. Gel Casting and Loading

Clean the glass plates and spacers with water and ethanol. Assemble the glass plates and spacers assembly and let dry. To orient and identify the gel, cut a small piece of filter paper to any recognizable shape (triangle, square, diamond, rectangle, pentagon, etc.) and drop it to the bottom of the gel assembly.

Mix the components of the separating gel, and pour the mixture in the assembly, leaving adequate empty space at the top to cast the subsequent stacking gel (typically 2–5 cm, depending on the size of the gel and of the anticipated sample volume). Overlay with 2 mm of water-saturated butanol and let polymerize (*see* **Note 5**). Once the gel is polymerized (c. 30 min), remove the upper liquid layer (butanol plus gel exudate) and replace with 5 mm of water. Protect from evaporation with parafilm, or transfer in a closed humid box (*see* **Note 6**). When needed, remove the upper water layer, mix the components of the staking gel and pour the gel mixture in the gel assembly. Insert the sample comb and let polymerize for 30 min. Remove the comb, and remove the unpolymerized liquid with a needle. Add cathode buffer to fill the sample wells, and load the samples in each well with a syringe or with a sample loading micropipet tip.

3.2. Gel Casting for Two-Dimensional Gel Electrophoresis

Gel casting proceeds as for one-dimensional gels, except that no stacking gel is needed. The separating gel mixture can the be cast up to 5 mm from the top, and overlaid with water-saturated butanol. The gel is then kept until use as described for one-dimensional gels. The IEF gels are equilibrated in the gel equilibration buffer for 5 min (carrier ampholytes tube gels) to 20 min (immobilized pH gradient strips).

When ready to use, melt the agarose, remove the water layer from the top of the gel, remove the equilibration buffer from the IEF gel, and insert the IEF gel on the top of the second dimension gel. Seal in place with agarose and let the agarose gel set.

3.3. Gel Running

Place the loaded gel in the electrophoresis tank. Fill the chambers with the adequate electrode buffer. The taurine-containing buffer must be at least in the cathode chamber (connected to the “minus” lead of the power supply). When separate electrode chambers with no buffer recirculation are present, a cheaper, glycine-containing anode electrode buffer can be used in the anode chamber (connected to the “plus” electrode of the power supply).

Gels can be run at constant voltage, power or current. We recommend to run first the gels at 25 V constant voltage for 30 to 60 min to ensure slow and complete protein entry into the gel. The gels can then be run at the maximum power that does not lead to overheating. This depends on the gel size paramaters (length; width and thickness) as well as on the type of cooling used.

We run 200 × 160 × 1.5 mm gels in a thermostated chamber with recirculating cooling water at 12 W/gel in this step.

3.4. Subsequent Processing

The migration is stopped when the bromophenol blue front, materializing the moving boundary, reaches the bottom of the separating gel. Gel made in this taurine system can be processed exactly as standard SDS gels cast in the glycine system. This includes staining with coomassie blue, fluorescent stains, silver nitrate or silver-ammonia stains, or blotting. Typical examples of the resolution obtained with the taurine gels, together with the tuning of the resolution window by buffer change, are shown in **Figs. 1** and **2**.

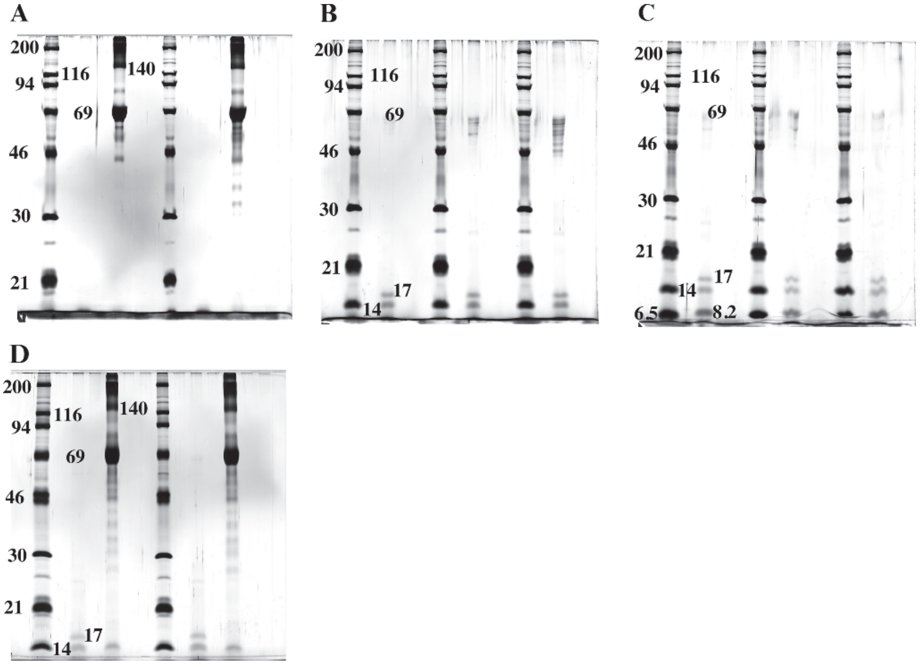

Fig. 1. Analysis of molecular weight markers. Molecular weight marker proteins were analysed by SDS-PAGE in various electrophoretic systems. The markers are: on the left lane, Bio-Rad wide range markers, in the middle lane, albumin + Myoglobin-CNBr peptides, and in the right lane, albumin crosslinked with pyromellitic dianhydride (on gels A and D only). The molecular weight is indicated close to the corresponding band. Proteins were detected by silver staining (nonammoniacal). **(A)** 10% gel, Tris taurine system (low speed). **(B)** 10% gel, Tris taurine system (medium speed). **(C)** 10% gel, Tris taurine system (high speed). **(D)** 10% gel, Tris glycine system pH 8.8.

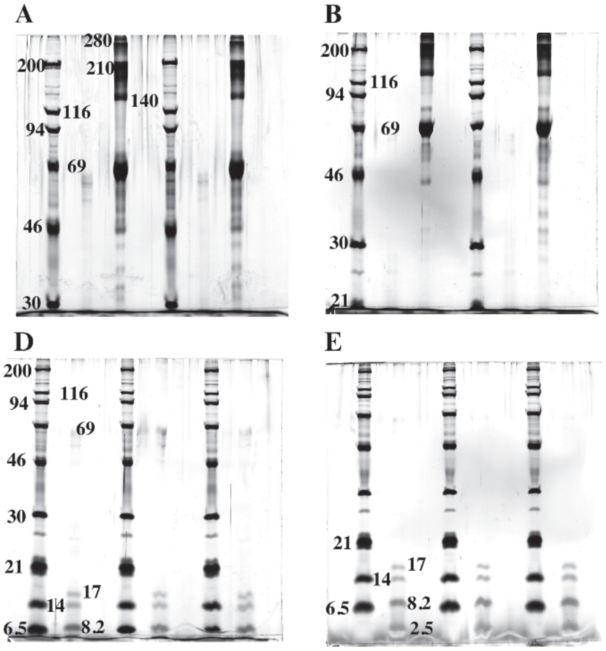

Fig. 2. Effect of double alteration of buffer and acrylamide concentration. The same molecular weight markers as in **Fig. 1** were used. **(A)** 7.5% gel, Tris taurine system (low speed). **(B)** 9% gel, Tris taurine system (low speed). **(C)** 10% gel, Tris taurine system (medium speed). **(D)** 10% gel, Tris taurine system (high speed). **(E)** 11.5% gel, Tris taurine system (high speed).

4. Notes

1. The versatility of the boundary speed means in turn that the pH of the gels buffers must be carefully controlled. This is especially difficult in the case of Tris buffers, because (i) many pH-meter electrodes have a sluggish response to Tris and (ii) Tris pK varies strongly with temperature (0.03 pH unit per °C). This means that the observed pH varies strongly with the laboratory temperature and/or the speed of addition of HCl. To avoid this variability, we recommend to make a buffer of constant composition, corresponding to the adequate pH. These buffers are most adequately made either by mixing Tris and Tris hydrochloride powders, or by mixing Tris powder with commercial titrated solutions of hydrochloric acid. The medium speed buffers has a moving boundary speed equivalent to the one of the Laemmli's buffer.

2. According to the theory of discontinuous electrophoresis, taurine is needed only in the cathode buffer, while Tris is required in the anode buffer. Thus, in an electrophoresis apparatus with separate buffer chambers, the cheaper glycine can replace taurine in the anode buffer. In an apparatus with a single chamber and/or with buffer recirculation, the taurine buffer must be used as electrode buffer. The electrode buffers are made the day of use, but can be stored for up to one week at room temperature
3. Thioglycerol is a substitute to the classical mercaptoethanol for disulfide reduction. Its efficiency is similar, but the odor is considerably weaker.
4. The recipe given yields a 10% separating gels. To adjust the acrylamide concentration, adjust the volume of stock acrylamide solution and complete to a total of 60ml (including the 10 ml of gel buffer) with water.
5. Polymerization is far from finished when the gel is set (i.e., converted from a liquid to a solid state). At this stage 50–70% of the monomer only is incorporated into the gel, depending on the temperature and time. Leaving the gel to "ripen" overnight at room temperature ensures >90% incorporation of monomers into the gel, leading to more consistent electrophoretic separations, less problems of reaction of proteins with acrylamide, and decreased safety problems with the toxic monomers
6. Once polymerized and "ripened," gels can be stored in a humid box for up to one week in the cold room.

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26

Cetyltrimethylammonium Bromide Discontinuous Gel Electrophoresis of Proteins

M_r-Based Separation of Proteins with Retained Native Activity

Robert E. Akins and Rocky S. Tuan

1. Introduction

This chapter describes a novel method of electrophoresis that allows the fine separation of proteins to be carried out with the retention of native activity. The system combines discontinuous gel electrophoresis in an arginine/*N*-Tris (hydroxymethyl methylglycine) (Tricine) buffer with sample solubilization in cetyltrimethylammonium bromide (CTAB). Because the components that distinguish this system are **C**TAB, **a**rginine, and **T**ricine and because CTAB is a **cat**ionic detergent, we refer to this method as CAT gel electrophoresis ***(1,2)***. Proteins separated on CAT gels appear as discrete bands, and their mobility is a logarithmic function of M_r across a broad range of molecular weights. After CAT electrophoresis, many proteins retain high enough levels of native activity to be detected, and gel bands may be detected by both M_r and protein-specific activities. In this chapter, we provide a description of the procedures for preparing and running CAT gels. We also provide some technical background information on the basic principles of CAT gel operation and some points to keep in mind when considering the CAT system.

1.1. Technical Background

The electrophoretic method of Laemmli ***(3)*** is among the most common of laboratory procedures. It is based on observations made by Shapiro et al. ***(4)*** and Weber and Osborn ***(5)***, which showed that sodium dodecyl sulfate (SDS) could be used for the separation of many proteins based on molecular size. In Laemmli's method, SDS solubilization was combined with a discontinuous gel system using a glycine/Tris buffer, as detailed by Ornstein ***(6)*** and Davis ***(7)***

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(*see* Chapter 21). Typically, SDS-discontinuous gel electrophoresis results in the dissociation of protein complexes into denatured subunits and separation of these subunits into discrete bands. Since the mobility of proteins on SDS gels is related to molecular size, many researchers have come to rely on SDS gels for the convenient assignment of protein subunit M_r.

Unfortunately, it is difficult to assess the biological activity of proteins treated with SDS: proteins prepared for SDS gel electrophoresis are dissociated from native complexes and are significantly denatured. Several proteins have been shown to renature to an active form after removal of SDS ***(8,9)***; however, this method is inconvenient and potentially unreliable. A preferred method for determining native protein activity after electrophoresis involves the use of nonionic detergents like Triton X 100 (Tx-100) ***(10)***; however, proteins do not separate based on molecular size. The assignment of M_r in the nonionic Tx-100 system requires the determination of mobilities at several different gel concentrations and "Ferguson analysis" ***(11–13)***. The CAT gel system combines the most useful aspects of the SDS and Tx-100 systems by allowing the separation of proteins based on M_r with the retention of native activity.

Previous studies have described the use of CTAB and the related detergent tetradecyltrimethylammonium bromide (TTAB), in electrophoretic procedures for the determination of M_r ***(14–18)***. In addition, as early as 1965, it was noted that certain proteins retained significant levels of enzymatic activity after solubilization in CTAB ***(19)***. A more recent report further demonstrated that some proteins even retained enzymatic activity after electrophoretic separation in CTAB ***(14)***. Based on the observed characteristics of CTAB and CTAB-based gel systems, we developed the CAT gel system.

In contrast to previous CTAB-based gel methods, the CAT system is discontinuous and allows proteins to be "stacked" prior to separation (*see* ***refs. 6*** and ***7***). CAT gel electrophoresis is a generally useful method for the separation of proteins with the retention of native activity. It is also an excellent alternative to SDS-based systems for the assignment of protein M_r (*see* **Note 1**).

1.2. Basic Principles of CAT Gel Operation

The CAT gel system is comprised of two gel matrices and several buffer components in sequence. A diagram of the CAT gel system is shown in **Fig. 1**. In an applied electric field, the positive charge of the CTAB–protein complexes causes them to migrate toward the negatively charged cathode at the bottom of the system. The arginine component of the tank buffer also migrates toward the cathode; however, arginine is a *zwitterion*, and its net charge is a function of pH. The arginine is positively charged at the pH values used in the tank buffer, but the pH values of the stacking gel and sample buffer are closer to the pI of arginine, and the arginine will have a correspondingly lower net

TANK BUFFER	--------ANODE--------- 25 mM Tricine pH 8.2 0.1% CTAB 14 mM Arginine Free Base
SAMPLE BUFFER	10 mM Tricine-NaOH pH 8.8 1% CTAB 10% Glycerol
STACKING GEL	0.7% Agarose 125 mM Tricine-NaOH pH 9.96 0.1% CTAB
SEPARATING GEL	6% Polyacrylamide 375 mM Tricine-NaOH pH 7.96
TANK BUFFER	25 mM Tricine pH 8.2 0.1% CTAB 14 mM Arginine Free Base -------CATHODE-------

Fig. 1. Diagram of a CAT gel. CAT gels begin at the top with the anode immersed in tank buffer and end at the bottom with the cathode immersed in additional tank buffer. The tank buffer solution contains CTAB, Arginine, and Tricine. Between the tank buffers are the stacking gel and the separating gel. The gels are made up of acrylamide polymers in a Tricine-NaOH-buffered solution. Prior to electrophoresis, protein samples are solubilized in a sample buffer that contains CTAB, to solubilize the protein sample, Tricine-NaOH, to maintain pH, and carry current and glycerol, to increase specific gravity. Proteins solubilized in sample buffer are typically layered under the upper tank buffer and directly onto the stacking gel. *See* **Note 3** for a listing of some physical characteristics of the CAT gel components.

positive charge as it migrates from the tank buffer into these areas. Therefore, the interface zone between the upper tank buffer and the stacking gel/sample buffer contains a region of high electric field strength where the sodium ions in the stacking gel/sample buffer (Tricine-NaOH) move ahead of the reduced mobility arginine ions (Tricine-arginine). In order to carry the electric current, the CTAB-coated proteins migrate more quickly in this interface zone than in the sodium-containing zone just below. As the interface advances, the proteins "stack," because the trailing edge of the applied sample catches up with the leading edge. When the cathodically migrating interface zone reaches the separating gel, the arginine once again becomes highly charged owing to a drop in the pH relative to the stacking gel. Because of the sieving action of the matrix, the compressed bands of stacked proteins differentially migrate through the separating gel based on size.

Two features of CTAB-based gels set them apart from standard SDS-based electrophoretic methods. First, proteins separated in CTAB gels migrate as a function of log M_r across a much broader range of molecular weights than do proteins separated in SDS gels. As shown in **Fig. 2**, a plot of relative migration distance, as a function of known log M_r of standard proteins, results in a straight line. Because of the consistent relationship between M_r and distance migrated,

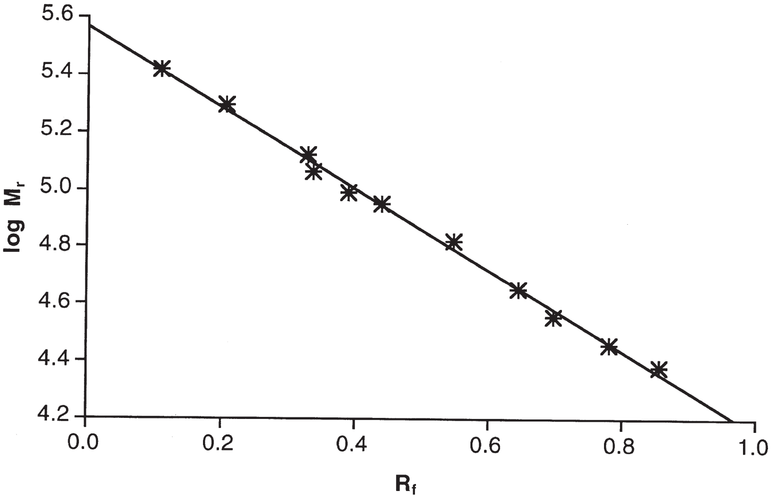

Fig. 2. Mobility of proteins in a CAT gel as a function of M_r. A mixture of proteins fractionated in a CAT gel with a 6% T acrylamide separator and a 0.7% agarose stacker was visualized by CBB R-250 staining. Relative mobilities (R_f) were calculated as distance, migrated divided by total distance to the salt/dye front and were plotted against the known M_r values for each protein band. The plot is linear across the entire range ($R^2 > 0.99$). Protein bands included trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phospate dehydrogenase (36 kDa), ovalbumin (45 kDa monomer and 90 kDa dimer), bovine serum albumin (66 kDa monomer and 132, 198, and 264 kDa multimers), phosphorylase-B (97.4 kDa), and β-galactosidase (116 kDa). *See* **Note 2** concerning the comparison of R_f values from different gels.

the relative molecular weights of unknown proteins can be determined. CAT gels may be especially useful for the assignment of M_r to small proteins or for the comparison of proteins with very different molecular weights. Second, the retention of significant levels of native activity in CAT gels allows electrophoretic profiles to be assessed *in situ* for native activities without additional steps to ensure protein renaturation (*see* **Note 2**). Taken together, these two characteristics of CAT gels make them an attractive alternative to standard electrophoretic systems.

2. Materials

1. CAT tank buffer: One liter of 5X tank buffer may be prepared using CTAB, Tricine, and arginine free base. First, prepare 80 mL of a 1 *M* arginine free base solution by dissolving 13.94 g in distilled water. Next, dissolve 22.40 g of Tricine in 900 mL of distilled water; add 5 g of CTAB, and stir until completely dissolved. Using the 1 *M* arginine solution, titrate the Tricine/CTAB solution until it reaches pH 8.2. Approx 75 mL of 1 *M* arginine solution will be required/L of CAT tank buffer. Since Tricine solutions change pH with changes in temperature, the tank buffer should be prepared at the expected temperature of use (typically 10–15°C). Finally, add distilled water to 1000 mL. Store the CAT tank buffer at room temperature. Prior to use, prepare 1X tank buffer by diluting 200 mL of the 5X stock to 1000 mL using distilled water of the appropriate temperature (usually 10–15°C); filter the 1X tank buffer through #1 Whatman filter paper to remove any particulate material. The 1X CAT tank buffer may be stored cold, but it should not be reused (*see* **Note 5**). Note that CTAB is corrosive, and care should be taken when handling CTAB powder or CTAB solutions: avoid inhalation or skin contact as advised by the supplier.
2. CAT stacking gel buffer: Prepare a 500 m*M* Tricine-NaOH by dissolving 22.4 g of Tricine in 200 mL of distilled water. Add NaOH until the pH of the solution reaches 10.0. Bring the solution to a total volume of 250 mL using distilled water. As with all Tricine solutions, the pH of CAT stacking gel buffer should be determined at the expected temperature of use. The CAT stacking gel buffer should be stored at room temperature to avoid any precipitation that may occur during long-term cold storage.
3. CAT separating gel buffer: Prepare a 1.5 *M* Tricine-NaOH solution by dissolving 134.4 g of Tricine in 400 mL of distilled water. Add NaOH until the pH of the solution reaches 8.0. Bring the solution to a total volume of 500 mL using distilled water. As with all Tricine solutions, the pH of CAT separating gel buffer should be determined at the expected temperature of use. The CAT separating gel buffer should be stored at room temperature to avoid any precipitation that may occur during long-term cold storage.
4. CAT sample buffer: Dilute 0.67 mL of CAT separating gel buffer to approx 80 mL with distilled water; to this add 10 mL of glycerol and 1 g of CTAB. Mix the solution until all the components are dissolved, and adjust the pH to 8.8 using NaOH.

Bring the solution to a final volume of 100 mL using distilled water. In some cases, it may be helpful to add a low-mol-wt cationic dye that will be visible during electrophoresis: 10 µL of a saturated aqueous solution of crystal violet may be added/mL of sample buffer. Note that CTAB is corrosive, and care should be taken when handling CTAB powder or CTAB solutions: avoid inhalation or skin contact as advised by the supplier. Store CAT sample buffer at room temperature to avoid precipitation of the components.

5. Acrylamide stock solution: A 40% acrylamide stock solution may be prepared by combining 38.93 g of ultrapure acrylamide with 1.07 g of *bis*-acrylamide in a total of 100 mL of distilled water. The final solution is 40%T (w/v) and 2.67%C (w/w). The "%T" and "%C" values indicate that the total amount of acrylamide in solution is 40 g/100 mL and that the amount of *bis*-acrylamide included is 2.67% of the total acrylamide by weight. The acrylamide stock solution should be stored in the refrigerator. Unpolymerized acrylamide is very toxic, and great care should be taken when handling acrylamide powders and solutions: Follow all precautions indicated by the supplier, including the wearing of gloves and a particle mask during preparation of acrylamide solutions.
6. Agarose stock solution: A ready-to-use agarose stacking gel solution may be prepared by combining 25 mL of CAT stacking gel buffer, 0.1 g CTAB, and 0.7 g of electrophoresis-grade agarose distilled to a final volume of 100 mL. Mix the components well, and, if necessary, adjust the pH to 10.0. Heat the solution in a microwave oven to melt the agarose, and swirl the solution to mix thoroughly. Divide the agarose stock solution into 10 aliquots, and store at 4°C until ready to use.
7. 10% Ammonium persulfate (AP): Dissolve 0.1 g of ammonium persulfate in 1 mL of distilled water. Make just prior to use.
8. Water saturated isobutanol: Combine equal volumes of isobutanol and distilled water. Mix well, and allow the two phases to separate: the water-saturated isobutanol will be the upper layer. Store at room temperature in a clear container so that the interface is visible.
9. CAT gel fixative: Combine 40 mL of distilled water, 10 mL of acetic acid, and 50 mL of methanol; mix well. Store CAT gel fixative in a tightly sealed container at room temperature.
10. Coomassie brilliant blue stain (CBB): Combine 40 mL of distilled water with 10 mL of acetic acid and 50 mL of methanol. Add 0.25 g of CBB R-250, and dissolve with stirring (usually overnight). Filter the solution through #1 Whatman paper to remove any particulate material. Store at room temperature in a tightly sealed container.
11. CBB Destain: Combine 437.5 mL of distilled water, 37.5 mL of acetic acid, and 25 mL of methanol. Mix well, and stored in a closed container at room temperature.
12. Electrophoresis apparatus: A suitable electrophoresis apparatus and power supply are required to run CAT gels. It is desirable to set aside combs, spacers, gel plates, and buffer tanks to use specifically with CAT gels; however, if the same apparatus

is to be used alternately for CAT gels and SDS gels, it is necessary to clean it thoroughly between each use. Often, the first CAT gel run in an apparatus dedicated to SDS gels will have a smeared appearance with indistinct bands. This smearing is the result of residual SDS, and subsequent CAT electrophoretic runs will resolve protein bands distinctly. This smearing may be somewhat avoided by soaking the gel apparatus and gel plates in CAT tank buffer prior to a final rinse in distilled water at the final step in the cleaning process.

The selection of an electrophoresis apparatus to be used for CAT gels should be based on a consideration of the electrical configuration of the system. Because molecular bromine (Br_2) will form at the anode, the anode should be located away from the top of the gel (*see* **Note 5**). In addition, it is important to realize that CAT gels are "upside-down" relative to SDS gels: proteins migrate to opposite electrodes in the two systems. Some electrophoresis apparatus are intentionally designed for use with SDS, and the anode (usually the electrode with the red lead) may be fixed at the bottom of the gel, whereas the cathode (usually the electrode with the black lead) is fixed at the top of the gel. If such an apparatus is used, the red lead wire should be plugged into the black outlet on the power supply, and the black lead should be plugged into the red outlet on the power supply. Crossing the wires in this fashion ensures that the CTAB-coated proteins in the CAT system will run into the gel and not into the tank buffer.

3. Method

The methods for the preparation and running of CAT gels are similar to other familiar electrophoretic techniques. In this section, we will describe the basic methods for preparing samples, casting gels, loading and running gels, visualizing protein bands, and transferring proteins to nitrocellulose (or other) membranes. We will emphasize the differences between CAT gels and other systems. To provide the best results, the recommendations of the manufacturer should be followed concerning the assembly of the apparatus and the casting of discontinuous gels.

3.1. Preparing Samples

1. Protein samples should be prepared at room temperature immediately prior to loading the gel. Typically, tissue fragments, cells, or protein pellets are resuspended in 1.5-mL microfuge tubes using CAT sample buffer (*see* **Note 6**). CAT sample buffer may also be used to solubilize cultured cells or minced tissues directly. In each case, the samples should be spun in a microfuge for 0.5 min at 16,000 *g* to pellet any debris or insoluble material prior to loading the gel. Good results have been obtained when the final concentration of protein in CAT sample buffer is between 1 and 5 mg/mL; however, the preferred concentration of protein will vary depending on the sample and the particular protein of interest. A series of protein dilutions should be done to determine the optimal solubilization conditions for a particular application.

3.2. Casting CAT Separating Gels

1. Assemble the gel plates and spacers in the gel casting stand as described by the manufacturer.
2. Prepare a separating gel solution by combining the 40%T acrylamide, CAT Separating gel buffer, and distilled water in the ratios indicated in Table 1. Mix the solution by swirling with the introduction of as little air as possible (oxygen inhibits the reactions necessary to accomplish acrylamide polymerization, *see* **Note 7**).
3. Degas the solution by applying a moderate vacuum for 5–10 min: the vacuum generated by an aspirator is generally sufficient.
4. Add 10% AP and TEMED to the solution as indicated in **Table 1**, and swirl the solution gently to mix. Note that insufficient mixing will result in the formation of a nonhomogeneous gel, but that vigorous mixing will introduce oxygen into the mixture.
5. Carefully pour the gel mixture into the gel plates to the desired volume; remember to leave room for the stacking gel and comb.
6. Finally, layer a small amount of water-saturated isobutanol onto the top of the gel. The isobutanol layer reduces the penetration of atmospheric oxygen into the surface of the gel and causes the formation of an even gel surface. Allow polymerization of the separating gel to proceed for at least 60 min to assure complete crosslinking; then pour off the isobutanol, and rinse the surface of the separating gel with distilled water.

3.3. Casting CAT Stacking Gels

Two different types of gel stackers are routinely used with CAT gels. For gel histochemical analyses, or where subsequent protein activity assays will be performed, stacking gels made from agarose have provided the best results.

1. Slowly melt a tube of agarose stock solution in a microwave oven; avoid vigorous heating of the solution, since boiling will cause foaming to occur and may result in air pockets in the finished gel.

Table 1
Preparation of Acrylamide Solutions for CAT Gels

Regent	4%T,mL	6%T,mL	8%T, mL	10%T,mL
40%T Acrylamide	1.00	1.50	2.00	2.50
Tricine buffer	2.50	2.50	2.50	2.50
Distilled water	6.39	5.89	5.39	4.89
Degas solution 10% AP	0.10	0.10	0.10	0.10
TEMED	0.01	0.01	0.01	0.01

Volumes indicated are in milliliters required to prepare 10 mL of the desired solution. Solutions should be degassed prior to the addition of the crosslinking agents, AP and TEMED.

2. Insert the gel comb into the apparatus, and cast the stacking gel directly onto the surface of the acrylamide separating gel. Allow the agarose to cool thoroughly before removing the comb (*see* **Note 8**).
3. As an alternative to agarose stacking gels, low%T acrylamide stackers may also be used. To prepare an acrylamide stacking gel, combine the 40%T acrylamide stock, CAT separating gel buffer (0.5 *M* Tricine-NaOH, pH 10.0), and distilled water in the ratios indicated in **Table 1**. Typically, a 4%T stacking gel is used. Degas the solution by applying a moderate vacuum for 5–10 min. Next, add 10% AP and TEMED to the solution as indicated in **Table 1**, and swirl the solution gently to mix. Insert the gel comb and cast the stacking gel directly onto the surface of the acrylamide separating gel. Do not use water-saturated isobutanol with stacking gels! It will accumulate between the comb and the gel, and cause poorly defined wells to form. Allow the stacking gel to polymerize completely before removing the comb.

3.4. Loading and Running CAT Gels

1. After the stacking and separating gels are completely polymerized, add 1X CAT tank buffer to the gel apparatus so that the gel wells are filled with buffer prior to adding the samples.
2. Next, using a Hamilton syringe (or other appropriate loading device), carefully layer the samples into the wells. Add the samples slowly and smoothly to avoid mixing them with the tank buffer, and fill any unused wells with CAT sample buffer. The amount of sample to load on a given gel depends on several factors: the size of the well, the concentration of protein in the sample, staining or detection method, and so forth. It is generally useful to run several dilutions of each sample to ensure optimal loading. Check that the electrophoresis apparatus has been assembled to the manufacturer’s specifications, and then attach the electrodes to a power supply. Remember that in CAT gels, proteins run toward the negative electrode, which is generally indicated by a black-colored receptacle on power supplies.
3. Turn the current on, and apply 100 V to the gel. For a single minigel (approx 80 mm across, 90 mm long, and 0.8 mm thick), 100 V will result in an initial current of approx 25 mA. Excessive current flow through the gel should be avoided, since it will cause heating.
4. When the front of the migrating system reaches the separating gel, turn the power supply up to 150 V until the front approaches the bottom of the gel. The total time to run a CAT gel should be around 45–60 min for minigels or 4–6 h for full-size gels.

3.5. Visualization of Proteins

1. As with any electrophoretic method, proteins run in CAT gels may be visualized by a variety of staining techniques. A simple method to stain for total protein may be carried out by first soaking the gel for 15 min in CAT gel fixative, followed by soaking the gel into CBB stain until it is thoroughly infiltrated. Infiltration can take

as little as 5 min for thin (0.8 mm), low-percentage (6%T) gels or as long as 1 h for thick (1.5 mm), high-percentage (12%T) gels. When the gel has a uniform deep blue appearance, it should be transferred to CBB destain.

2. Destain the gel until protein bands are clearly visible (*see* **Note 9**). It is necessary to observe the gel periodically during the destaining procedure, since the destain will eventually remove dye from the protein bands as well as the background. Optimally, CBB staining by this method will detect about 0.1–0.5 µg of protein/protein track; when necessary, gels containing low amounts of total protein may be silver-stained (*see* **Note 10**). Note that the CBB stain may be retained and stored in a closed container for reuse.
3. In addition to total protein staining by the CBB method, enzyme activities may be detected by a variety of histochemical methods. The individual protocol for protein or enzyme detection will, of course, vary depending on the selected assay (*see* **Note 11**). Generally, when CAT gels are to be stained for enzyme activity, they should be rinsed in the specific reaction buffer prior to the addition of substrate or detection reagent. The CAT gel system provides an extremely flexible method for the analysis of protein mixtures by a variety of direct and indirect gel staining methods.

3.6. Electrophoretic Transfer Blotting

Similar to other methods of electroblotting (e.g., *see* Chapters 57 and 58), proteins can be transferred from CAT gels to polyvinylidene difluoride (PVDF) membranes. Blotting CAT gels is similar to the standard methods used for SDS-based gels with the notable difference that the current flow is reversed. We have successfully transferred proteins using the method described in Chapter 58 with the following changes: 1) A solution of 80% CAT gel running buffer and 20% MeOH was used as the transfer buffer, 2) the transfer membrane was PVDF, and 3) the polarity of the apparatus was reversed to account for the cationic charge of the CTAB. ***(2)*** As with electrophoresis, the tank and apparatus used for electroblotting should be dedicated to CTAB gels (*see* **Note 9**).

3.7. Conclusion

Gel electrophoresis of proteins is a powerful and flexible technique. There are many excellent references for general information concerning the theory behind electrophoresis. One good source of information is Hames and Rickwood ***(25)***. The CAT gel electrophoresis system presented here efficiently stacks and separates a wide range of proteins as a function of M_r and preserves native enzymatic activity to such a degree that it allows the identification of protein bands based on native activity. The nature of the interaction between CTAB and native proteins allows the formation of complexes in which the amount of CTAB present is related to the size of the protein moiety. Based on characterizations of detergent/protein interactions that indicate consistent levels of detergent binding without massive denaturation ***(10,26–28)***, the retention of activity in certain detergent/protein complexes is expected, and CTAB is likely to represent a class of ionic detergents that allow the electrophoretic separation of native proteins by M_r. Since the level of retained activity after solubilization in CTAB varies depending on the protein of interest, and a given protein will retain varying amounts of measurable activity depending on the detergent used (*see* **ref. 1**, for example), a battery of detergents may be tried prior to selecting the desired electrophoresis system. In general, cationic detergents (e.g., the quartenary ammoniums like CTAB, TTAB, and so forth) may be used in the arginine/Tricine buffer system described above; anionic detergents (e.g., alkyl sulfates and sulfonates) may be substituted for SDS in the familiar glycine/Tris buffer system ***(29)***.

The CAT gel system and its related cationic and anionic gel systems provide useful adjuncts to existing biochemical techniques. These systems allow the electrophoretic separation of proteins based on log M_r with the retention of native activity. The ability to detect native protein activities, binding characteristics, or associations and to assign accurate M_r values in a single procedure greatly enhances the ability of researchers to analyze proteins and protein mixtures.

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4. Notes

1. SDS vs CTAB: CTAB and SDS are very different detergents. CTAB is a cationic detergent, and proteins solubilized in CTAB are positively charged; SDS is an anionic detergent, and proteins solubilized in SDS are negatively charged. In terms of electrophoretic migration, proteins in CTAB gels migrate toward the cathode (black electrode), and proteins in SDS gels run toward the anode (red electrode). SDS is not compatible with the CAT gel system, and samples previously prepared for SDS-PAGE are not suitable for subsequent CAT gel electrophoresis. Also, the buffer components of the typical SDS-PAGE system, Tris and glycine, are not compatible with the CAT system: Tricine and arginine should be used with CAT gels.

   Although the detergents are different, protein banding patterns seen in CAT gels are generally similar to those seen when using SDS-PAGE. R_f values of proteins fractionated by CAT electrophoresis are consistently lower than R_f values determined on the same%T SDS-PAGE, i.e., a particular protein will run nearer the top of a CAT gel than it does in a similar

%T SDS gel. As a rule of thumb, a CAT gel with a 4%T stacker and a 6%T separator results in electrophoretograms similar to an SDS gel with a 4%T stacker and 8%T separator. Differences between the protein banding patterns seen in CAT gels and SDS gels are usually attributable to subunit associations: multisubunit or self-associating proteins are dissociated to a higher degree in SDS than in CTAB, and multimeric forms are more commonly seen in CAT gels than in SDS gels.

2. Detergent solubilization and protein activity: Many proteins separated on CAT gels may be subsequently identified based on native activity, and under the conditions presented here, CTAB may be considered a nondenaturing detergent. Denaturants generally alter the native conformation of proteins to such an extent that activity is abolished; such is the case when using high levels of SDS. Sample preparation for SDS-based gels typically results in a binding of 1.4 g of SDS/1 g of denatured protein across many types of proteins ***(20)***, and it is this consistent ratio that allows proteins to be electrophoretically separated by log M_r. Interestingly, at lower concentrations of SDS, another stable protein binding state also exists (0.4 g/1 g of protein) which reportedly does not cause massive protein denaturation ***(20)***. In fact, Tyagi et al. ***(21)*** have shown that low amounts of SDS (0.02%) combined with pore-limit electrophoresis could be used for the simultaneous determination of M_r and native activity. The existence of detergent–protein complexes, which exhibit consistent binding ratios without protein denaturation, represents an exciting prospect for the development of new electrophoretic techniques: protein M_r and activity may be identified by any of a variety of methods selected for applicability to a specific system.
3. Comparing CAT gels: The comparison of different CAT gel electrophoretograms depends on using the same separating gel, stacking gel, and sample buffer for each determination. An increase in the acrylamide%T in the separating gel will cause bands to shift toward the top of the gel, and high%T gels are more suitable for the separation of low M_r proteins. In addition, the use of acrylamide or high-percentage agarose stackers will lead to the determination of R_f values that are internally consistent, but uniformly lower than those determined in an identical gel with a low-percentage agarose stacker. This effect is likely owing to some separation of proteins in the stacking gel, but, nonetheless, to compare R_f values among CAT gels, the stacker of each gel should be the same. Similarly, any changes in sample preparation (for example, heating the sample before loading to dissociate protein subunits) or the sample buffer used (for example, the addition of salt or urea to increase sample solubilization) often precludes direct comparisons to standard CAT gel electrophoretograms.
4. Characteristics of system components: The CAT gel system is designed around the detergent CTAB. The other system components were selected

based on the cationic charge of CTAB and the desire to operate the gel near neutral pH. Some of the important physical characteristics of system components are summarized here; the values reported are from information supplied by manufacturers and *Data for Biochemical Research* **(22)**.

In solution, CTAB exists in both monomer and micelle forms. CTAB has a monomer mol wt of 365 Dalton. At room temperature and in low-ionic-strength solutions ($<0.05\,M$ Na^+), CTAB has a critical micelle concentration (CMC) of about 0.04% (≈ 1 m*M*). The CMC may be defined as the concentration of detergent monomer that may be achieved in solution before micellization occurs; it depends on temperature, ionic strength, pH, and the presence of other solutes. The actual CMC of CTAB in the solutions used in the CAT gel system is not known. In low-ionic-strength solutions ($< 0.1\,M$ Na^+), the mol wt of CTAB micelles is approx 62 kDa with about 170 monomers/micelle. The solubility of CTAB is also a function of ionic conditions and solution temperature, and CTAB will precipitate from CAT sample buffer at temperatures below 10–15°C.

Arginine is an amino acid used in CAT tank buffer to carry current toward the cathode. Arginine is a zwitterion. It has a mol wt of 174.2 Dalton and contains three pH-sensitive charge groups ($pK_a = 1.8, 9.0, 12.5$) with an isoelectric point (pI) near pH 10.8. Arginine was selected as the zwitterionic stacking agent because of its basic pI: at near-neutral pH levels, arginine will be positively charged and will migrate toward the cathode when an electric current is applied. Arginine free base is used in CAT tank buffer, and the proper pH of the CAT tank buffer is arrived at by mixing an acidic solution of Tricine with a basic solution of arginine.

Tricine functions to maintain the desired pH levels throughout the system. Tricine has a mol wt of 179.2 Dalton, and a pK_a of 8.15 (there is also a second $pK_a \approx 3$). During electrophoresis, Tricine also functions as a counterion to carry current toward the anode during electrophoresis. Tricine-buffered solutions will tend to change pH as temperature changes (a drop in temperature from 25 to 4°C will result in a shift in pH of about 0.5 U). Also, at the pH used in CAT stacking gels and CAT sample buffer, Tricine has a relatively low buffering capacity; therefore, the pH of the stacker and sample buffer should be confirmed, especially when any additions or alterations are made to the standard recipes.

5. Bromine drip: CTAB is an ionic compound comprised of both cetyltrimethylammonium cations and bromide anions. During electrophoresis, bromide anions migrate toward the anode at the top of the CAT system. Since electrons are removed at the anode, molecular bromine (Br_2) is formed at the anode. Under standard conditions, Br_2 is a dense, highly reactive liquid. While running CAT gels, a small amount of Br_2 will drip from the anode, and, if the anode is located directly above the top of the gel, Br_2 may drip

onto the samples. The "bromine drip" problem may be avoided if the apparatus used in CAT gel electrophoresis is configured so that the anode is away from (or even below) the top of the gel. Substitution of the bromide anion during CTAB preparation (perhaps with chloride to make CTACl) would be useful; however, the authors know of no high-quality commercial source. It should also be noted that the accumulation of reactive Br_2 during electrophoresis may preclude the reuse of tank buffer.

6. CAT sample buffer: The CTAB component of CAT sample buffer precipitates at low temperature (below 10–15°C). Samples should be prepared at room temperature immediately prior to use. Protease inhibitors, for example, phenylmethylsulfonyl fluoride (PMSF), may be added to the sample buffer to inhibit endogenous protease activity. The potential effects of any sample buffer additives (including PMSF) on the enzyme of interest should be assessed in solution before using the additive. Also, to avoid contaminating samples with "finger proteins" from the experimenter's hands, gloves should be worn when handling samples or sample buffer.
7. Acrylamide CAT gels: The polymerization of acrylamide generally involves the production of acrylamide free radicals by the combined action of ammonium persulfate and TEMED. Oxygen inhibits acrylamide polymerization by acting as a trap for the ammonium persulfate and TEMED intermediate free radicals that are necessary to accomplish chemical crosslinking. It is important to degas acrylamide solutions prior to the addition of crosslinking agents. CTAB itself interferes with gel polymerization as well and should not be included with acrylamide solutions; in the case of agarose stacking gels, however, CTAB may be included. Also, we have noticed a slight increase in the time required for acrylamide polymerization in the presence of Tricine buffer, but Tricine does not apparently affect gel performance. Although gelation will occur in about 10–20 min, polymerization should be allowed to go to completion (1–2 h) before running the gel. Finally, to optimize gel performance and reproducibility, both degassing and polymerization should be done at room temperature: cooling a polymerizing gel does not accelerate gel formation, and warming a polymerizing gel may cause brittle matrices to form.

   The presence of residual crosslinking agents in the acrylamide matrix may result in the inhibition of some protein activities. An alternative method of gel formation that avoids the use of reactive chemical crosslinkers involves photoactivation of riboflavin to initiate acrylamide polymerization (*see* **ref. 23**). We have had success using acrylamide separating gels polymerized with ammonium persulfate and TEMED in combination with agarose stacking gels; however, in some instances, riboflavin polymerized gels may be useful.

8. Agarose CAT gels: When using agarose stackers, remove the comb carefully to avoid creating a partial vacuum in the wells. To avoid pulling the agarose stacker away from the underlying acrylamide separating gel, wiggle the comb so that air or liquid fills the sample wells as the comb is lifted out.
9. Smeared gels: During electrophoresis, proteins run as mixed micelles combined with CTAB and other solution components. The presence of other detergents or high levels of lipid in the sample solution may result in a heterogenous population of protein-containing micelles. In such cases, protein bands may appear indistinct or smeared. Samples that have been previously solubilized in another detergent or that contain high levels of lipid may not be suitable for subsequent CAT gel analysis. Also, precipitation may occur when CTAB is mixed with polyanions (e.g., SDS micelles or nucleic acids). Samples containing high levels of nucleic acid or SDS may not be suitable for CAT gel analysis owing to precipitation in the sample buffer. Furthermore, if the gel apparatus to be used for CAT gels is also routinely used for SDS-based gels, the apparatus should be thoroughly cleaned to remove traces of SDS prior to CAT gel analysis, especially from the gel plates. Interaction between SDS and CTAB during electrophoresis may cause the formation of heterogenous mixed micelles or the precipitation of the components, and will invariably result in smeared gels. Although it is not always possible, it is preferable to have a separate apparatus dedicated to CAT gel use in order to avoid this problem.
10. Staining gels and transfer membranes: In addition to the basic CBB staining of protein bands, CAT gels may be stained by a variety of other techniques. Silver staining ***(24)*** allows the detection of even trace amounts of proteins (1–10 ng), including any fingerprints or smudges that would be otherwise undetected. In silver staining it is, therefore, essential to wear gloves throughout the procedure, even when cleaning the glassware prior to assembly of the apparatus. When the CAT gel is done, stain it with CBB to visualize proteins. (It is a good idea to take a photograph of the gel at this point, but be careful when handling it to avoid smudging the surface.) Next, place the gel into a 10% glutaraldehyde solution and soak in a fume hood for 30 min with gentle shaking. Rinse the gel well with distilled water for at least 2 h (preferably overnight) changing the water frequently; the gel will swell and become soft and somewhat difficult to handle while it is in the water. Combine 1.4 mL NH_4OH (concentrated solution ≈ 14.8 *M*) and 21.0 mL 0.36% NaOH (made fresh); add approx 4.0 mL of 19.4% $AgNO_3$ (made fresh) with constant swirling. A brown precipitate will form as the $AgNO_3$ is added, but it will quickly disappear; if it does not, a small amount of additional NH_4OH (just enough to dissolve the precipitate) may be added. Soak the gel in the ammoniacal/silver

solution for 10 min with shaking. Pour off the ammoniacal/silver solution and precipitate the silver with HCl. Transfer the gel to a fresh dish of distilled water and rinse for 5 min with two or three changes of water. Decant the water and add a freshly prepared solution of 0.005% citric acid and 0.019% formaldehyde (commercial preparations of formaldehyde are 37% solutions); bands will become visible at this point. Stop the silver-staining reaction by quickly rinsing the gel in water followed by soaking in a solution of 10% acetic acid and 20% methanol. Silver-staining takes a little practice to do well.

Transfer blots may be stained by a variety of conventional techniques. Two rapid and simple procedures are as follows: **Step (1)** Rinse the membrane briefly in phosphate-buffered saline containing 0.1% Tween 20 (PBS/Tw); soak the membrane in 0.1% solution of India ink in PBS/Tw until bands are detected (10 to 15 min.); rinse the blot in PBS/Tw. **Step (2)** Rinse the membrane briefly in phosphate-buffered saline (PBS); soak the blot in 0.2% Ponceau-S (3-hydroxy-4-(2-sulfo-4(sulfo-phenylazo) phenylazo)-2,7-naphthalene disulfonic acid) in 3% trichloroacetic acid and 3% sulfosalicylic acid for 10 min.; rinse the blot in PBS until bands appear. Ponceau S can be stored as a 10X stock solution and diluted with distilled water just prior to use. Staining with Ponceau-S is reversible, so blots should be marked at the position of mol-wt markers before continuing. When higher sensitivity is required to visualize bands, transfer membranes may be stained with ISS Gold Blot (Integrated Separation Systems, Natick, MA.

11. Enzyme activities and protein banding patterns: The histochemical detection of proteins in CAT gels is generally a straightforward process of soaking the gel in reaction buffer, so that the necessary compounds penetrate the gel, followed by the addition of substrate; however, not all enzymes retain detectable levels of activity when solubilized in CTAB or when they are run on CAT gels. It is a good idea to check the relative activity of the protein of interest in a CTAB-containing solution vs standard reaction buffer prior to investing time and effort into running a CAT gel. Of the proteins that do retain detectable the levels of activity, there are substantial differences in the level of retained activity after solubilization in CTAB. There is also the possibility that the protein of interest will run anomalously in CAT gels. Anomalous migration may occur owing to differential CTAB binding, conformational differences in the protein/CTAB mixed micelle, or the presence of previously unrecognized subunits or associated proteins. Unexpected enzyme histochemistry patterns in samples with the expected CBB protein staining pattern may reflect the presence of cofactors or other protein/protein interactions that are necessary for activity and should be interpreted accordingly.

It should also be pointed out that the intensity of a histochemical or binding assay does not necessarily reflect the actual level of enzyme in the sample. The rate of histochemical reaction within the gel matrix is not necessarily linear with respect to the amount of enzyme present: measured product also depends on the amount of substrate present and the ratio of product to substrate over time as well as the response of the detection apparatus. Reactions should be performed with an excess of substrate at the outset and should preferably be calibrated based on the varying level of a standard activity. Also, as with all detergents, CTAB may differentially solubilize proteins in a given sample, depending on the overall solution conditions. The detergent/protein solution is a complex equilibrium that may be shifted, depending on the level of other materials (proteins, lipids, salts, and so on) in the solution. When seeking to compare protein profiles on CAT gels, it is advisable to prepare samples from the same initial buffer and to solubilize at a uniform CTAB-to-protein ratio.

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Acetic Acid–Urea Polyacrylamide Gel Electrophoresis of Basic Proteins

Jakob H. Waterborg

1. Introduction

Panyim and Chalkley described in 1969 a continuous acetic acid–urea (AU) gel system that could separate very similar basic proteins based on differences in size and effective charge ***(1)***. For instance, unmodified histone H4 can be separated from its monoacetylated or monophosphorylated forms ***(2)***. At the acidic pH 3 of this gel system, basic proteins with a high isoelectric point will clearly have a net positive charge that will be the major determinant of electrophoretic mobility. If a single of these positive charges is removed, for example, by in vivo acetylation of one of the positively charged ε-amino lysine side chain residues in the small histone H4 protein (102 residues), a significant decrease in effective gel mobility is observed. Similarly, addition of a phosphate moiety decreases the net positive charge of the protein during gel electrophoresis by one. Separation between similarly sized and charged proteins, for example, the partially acetylated H2A, H2B, and H3 histones of most organisms, can typically be achieved only by inclusion of a nonionic detergent such as Triton X-100 (*see* Chapter 28).

In 1980, Bonner and co-workers introduced a discontinuous acetic acid–urea–Triton (AUT) variation that avoids the necessity for exhaustive preelectrophoresis ***(3)***, prevents deformation of sample wells ***(4)*** and generally produces much sharper, straighter bands. Omission of Triton from this method creates the high capacity and high resolution AU gel electrophoresis protocol described in **Subheading 3**. **Figure 1** shows an example of the possibilities and limitations of the AU gel system. Yeast histones were extracted from four parallel cultures using a novel method that preserves all postsynthetic modifications ***(5)***. The separation of single-charge differences, proven to be caused by acetylation of

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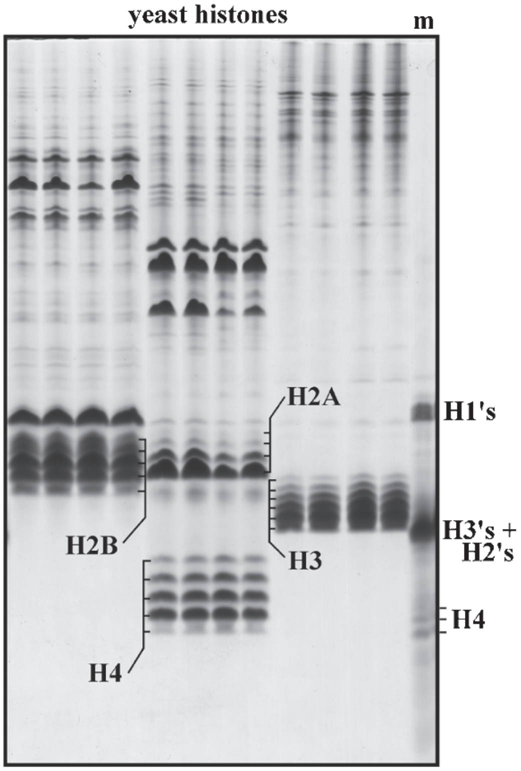

Fig. 1. Histones of the yeast *Saccharomyces cerevisiae* were extracted from crude nuclei, prepared in such a way that protein integrity and postsynthetic modifications were maintained ***(5)***. Histones from four parallel cultures (approx 2 × 10^{10} cells) were extracted, fractionated by reversed-phase HPLC into pools containing histone H2B (*left four lanes*), coeluting histone H2A and H4 (*center four lanes*) and histone H3 (*right four lanes*). In each histone species, the fastest mobility band represents nonacetylated histone. In each slower moving species one more lysine has been acetylated, neutralizing the positive charge of the lysine side chain, reducing the protein gel mobility by one charge. Virtually all low-mobility nonhistone proteins in this gel can be removed from yeast histone preparations by 100,000 mol wt ultrafiltration ***(5)***. The "m" indicates a marker lane with total calf thymus histones. Calf histone species are marked along the side. Note the closely overlapping pattern of H2A, H2B, and H3 histones. The long AU separating gel was 1 mm thick, 19 cm wide, and 27 cm long. The top of the gel is visible at the top of the figure. Electrophoresis was at 250 V constant voltage for 20 h with electrical current decreasing from 22 mA to 6 mA. The electrophoretic front, visualized using methylene blue, was below the lower edge of the figure, close to the lower margin of the gel. The gel was stained overnight in Coomassie, destained in 4 h with two aliquots of polyurethane foam, and digitized under standard conditions on a UMAX Powerlook II flatbed scanner with transilluminator.

lysines, is clearly demonstrated for each histone species (**Fig. 1**). It is clear that, without fractionation, the patterns of H2B, H2A, and H3 histones would overlap, preventing quantitation of each protein and its acetylation. Protein band shapes are generally sharp and straight. Compression of band shapes, visible for some high-abundance nonhistone proteins with lower gel mobilities shown in **Fig. 1**, can be minimized by decreasing the protein concentration during gel stacking by increasing gel thickness.

AU gels are currently used for very dissimilar proteins with isoelectric points lower than those of histones but sufficiently above pH 3 to be positively charged during gel electrophoresis. Examples include neutrophil defensins ***(6)***, antimicrobiol nasal secretions ***(7)***, enzymes like tyrosinase ***(8)***, serum isoenzymes ***(9)***, chemokines ***(10)*** and basic protamines ***(11)***. Typically curved, somewhat diffuse protein bands, characteristic for native, non-stacking electrophoretic gel separations are obtained. The superior discontinuous gel system described here has only been used for histones, producing clearer bands in a shorter period of time than achievable in continuous AU gel modes ***(1–2)***.

2. Materials

1. Vertical gel apparatus for short (15 cm) or long (30 cm) slab gels. A gel electrophoresis apparatus that allows gel polymerization between the glass plates with spacers, without necessarily being assembled in the apparatus, is preferable. This facilitates the even and complete photopolymerization of the acrylamide gel. In this type of apparatus the glass–gel sandwich is typically clamped to the lower buffer reservoir, which acts as a stand, after which the upper buffer reservoir is clamped to the top of the gel assembly.

   Details of the procedure are described for a fairly standard and flexible gel apparatus that uses two rectangular glass plates (4 mm thick standard plate glass with sanded edges), 21 cm wide and 32.5 and 35.5 cm long, respectively. The Plexiglas bottom buffer reservoir with platinum electrode is 22.5 cm wide with three sides 5 cm high and one of the long sides 12.5 cm high. The glass plates are clamped to this side. The upper buffer reservoir with platinum electrode and a similar buffer capacity is 18 cm wide with one long side enlarged to measure 21 cm wide by 10 cm high. It contains a cutout of 18 cm wide and 3.5 cm high that allows access of the upper reservoir buffer to the top of the gel. The 21-cm-wide Plexiglas plate is masked with 5-mm-thick closed-cell neoprene tape (weather strip) and provides a clamping ridge for attachment to the top of the glass-gel sandwich.
2. Spacers and combs are cut from 1-mm Teflon sheeting. High-efficiency fluorography may benefit from 0.5-mm spacers. Teflon up to 3 mm thick is less easy to cut but yields very high capacity gels (*see* **Note 1**).

   Two side spacers (1.5 × 35 cm) and one bottom spacer (0.5 × 24 cm) are required. Added to the top of the side spacers is 3 cm adhesive, closed-cell neoprene tape (weather strip, 14 mm wide and 5 mm thick). This is not required if a more expensive glass plate with “rabbit ears” is used instead of the rectangular shorter plate.

Combs have teeth 5–10 mm wide and 25–50 mm long, separated by gaps of at least 2.5 mm. For the detailed protocol described, a 15-cm-wide comb with 20 teeth of 5 × 30 mm is used.

3. Vaseline pure petroleum jelly.
4. Acrylamide stock solution: 60% (w/v) acrylamide, highest quality available, in water (*see* **Note 2**). The acrylamide is dissolved by stirring. Application of heat should be avoided, if possible, to prevent generating acrylic acid. The solution can be kept at least for 3 mo on the laboratory shelf at room temperature. Storage at 4°C can exceed 2 yr without detectable effects.
5. *N,N′* -methylene bis-acrylamide stock solution: 2.5% (w/v) in water (*see* **Note 2**).
6. Glacial acetic acid (HAc): 17.5 *M*.
7. Concentrated ammonium hydroxide: NH_4OH, 28–30%, approx 15 *M*.
8. *N, N, N′*, *N′* -Tetramethylenediamine (TEMED), stored at 4°C.
9. Riboflavin-5′ -phosphate (R5P) solution: 0.006% (w/v) in water. This solution is stable for more than 6 mo if kept dark and stored at 4°C.
10. Urea, ultrapure quality.
11. Side-arm suction flasks with stoppers, magnetic stirrer, stirrer bar, and water-aspirator vacuum; measuring cylinders with silicon-rubber stoppers; pipets, and pipetting bulbs or mechanical pipetting aids; 1- and 5-mL plastic syringes, with 20-gauge needles.
12. Fluorescent light box with diffuser for even light output and with the possibility to stand vertically. Light intensity should equal or exceed 5 klx at a distance of 5–10 cm. A high-quality X-ray viewing light box with three 40-W bulbs typically will meet this specification.
13. Aluminum foil.
14. Electrophoresis power supply with constant voltage mode at 300–500 V with up to 50 mA current, preferably with a constant power mode option.
15. Urea stock solution: 8 *M* urea in water. An aliquot of 40 mL with 1 g of mixed-bed resin (Bio-Rad [Hercules, CA] AG 501-X8) can be used repeatedly over a period of months if refrozen and stored between use at −20°C (*see* **Note 3**).
16. Phenolphthalein indicator solution: 1% (w/v) in 95% ethanol, stored indefinitely at room temperature in a closed tube.
17. Dithiothreitol (DTT, Cleland's reagent) is stored at 4°C and is weighed freshly for each use.
18. Methylene blue running front indicator dye solution: 2% (w/v) in sample buffer (*see* **Subheading 3.1.**, **step 22**).
19. Reference histones: Total calf thymus histones (Worthington, Freehold, NJ), stored dry at 4°C indefinitely or in solution at −80°C in 50-µL aliquots of 5 mg/mL in water for more than 1 yr (*see* **Subheading 3.1.**, **step 23**).
20. Glass Hamilton microsyringe (100 µL) with Teflon-tipped plunger.
21. Electrophoresis buffer: 1 *M* acetic acid, 0.1 *M* glycine (*see* **Note 4**). This solution can be made in bulk and stored indefinitely at room temperature.
22. Destaining solution: 20% (v/v) methanol, 7% (v/v) acetic acid in water.
23. Staining solution: Dissolve a fresh 0.5 g of Coomassie Brilliant Blue R250 in 500 mL of destaining solution for overnight gel staining (*see* **Note 5**). For rapid

staining within the hour the dye concentration should be increased to 1% (w/v). If the dye dissolves incompletely, the solution should be filtered through Whatman no. 1 paper to prevent staining artifacts.

24. Glass tray for gel staining and destaining.
25. Rotary or alternating table top shaker.
26. Destaining aids: Polyurethane foam for Coomassie-stained gels or Bio-Rad ion-exchange resin AG1-X8 (20–50-mesh) for Amido Black-stained gels.

3. Method

1. Assemble a sandwich of two clean glass plates with two side spacers and a bottom spacer, lightly greased with Vaseline to obtain a good seal, clamped along all sides with 2-in binder clamps. The triangular shape of these clamps facilitates the vertical, freestanding position of the gel assembly a few centimeters in front of the vertical light box.
2. Separating gel solution: Pipet into a 100-mL measuring cylinder 17.5 mL of acrylamide stock solution, 2.8 mL of *bis*-acrylamide stock solution, 4.2 mL of glacial acetic acid, and 0.23 mL of concentrated ammonium hydroxide (*see* **Notes 2** and **6**).
3. Add 33.6 g of urea and add distilled water to a total volume of 65 mL.
4. Stopper the measuring cylinder, and place on a rotary mixer until all urea has dissolved. Add water to 65 mL, if necessary.
5. Transfer this solution to a 200-mL sidearm flask with magnetic stir bar on a magnetic stirrer. While stirring vigorously, stopper the flask and apply water-aspirator vacuum. Initially, a cloud of small bubbles of dissolved gas arises, which clears after just a few seconds. Terminate vacuum immediately to prevent excessive loss of ammonia.
6. Add 0.35 mL of TEMED and 4.67 mL of R5P (*see* **Note 7**), mix, and pipet immediately between the glass plates to a marking line 5 cm below the top of the shorter plate (*see* **Notes 2** and **8**).
7. Carefully apply 1 mL of distilled water from a 1-mL syringe with needle along one of the glass plates to the top of the separating gel solution to obtain a flat separation surface.
8. Switch the light box on, and place a reflective layer of aluminum foil behind the gel to increase light intensity and homogeneity (*see* **Note 1**). Gel polymerization becomes detectable within 2 min and is complete in 15–30 min.
9. Switch the light box off, completely drain the water from between the plates, and insert the comb 2.5 cm between the glass plates. The tops of the teeth should always remain above the top of the short glass plate.
10. Stacking gel solution, made in parallel to **steps 2–4**: Into a 25-mL measuring cylinder, pipet 1.34 mL of acrylamide stock solution, 1.28 mL of *bis*-acrylamide stock solution, 1.14 mL of glacial acetic acid, and 0.07 mL of concentrated ammonium hydroxide (*see* **Notes 2** and **6**).
11. Add 9.6 g of urea, and add distilled water to a total volume of 18.6 mL.
12. Stopper the measuring cylinder, and place on a rotary mixer until all urea has dissolved. Add water to 18.6 mL, if necessary.

13. Once the separating gel has polymerized, transfer the stacking gel solution to a 50-mL sidearm flask with stir bar and degas as described under **step 5**.
14. Add 0.1 mL of TEMED and 1.3 mL of R5P, mix, and pipet between the plates between the comb teeth. Displace air bubbles.
15. Switch the light box on, and allow complete gel polymerization in 30–60 min.
16. Prepare sample buffer freshly when the separation gel is polymerizing (**step 8**). The preferred protein sample is a salt-free lyophilisate (*see* **Note 9**). Determine the approximate volume of sample buffer required, depending on the number of samples.
17. Weigh DTT into a sample buffer preparation tube for a final concentration of 1 *M*, that is, 7.7 mg/mL.
18. Per 7.7 mg of DTT add 0.9 mL of 8 *M* urea stock solution, 0.05 mL of phenolphtalein, and 0.05 mL of NH_4OH to the tube to obtain the intensely pink sample buffer.
19. Add 0.05 mL of sample buffer/sample tube with lyophilized protein to be analyzed in one gel lane (*see* **Note 10**). To ensure full reduction of all proteins by DTT, the pH must be above 8.0. If the pink phenolphtalein color disappears owing to residual acid in the sample, a few microliters of concentrated ammonium hydroxide should be added to reach an alkaline pH.
20. Limit the time for sample solubilization and reduction to 5 min at room temperature to minimize the possibility of protein modification at alkaline pH by reactive urea side reactions, for example, by modification of cysteine residues by cyanate.
21. Acidify the sample by adding 1/20 volume of glacial acetic acid.
22. To each sample add 2 μL of methylene blue running front dye (*see* **Note 11**).
23. Prepare appropriate reference protein samples: To 2 and 6 μL of reference histone solution with 10 and 30 μg total calf thymus histones, add 40 μL of sample buffer (**step 18**), 2.5 μL of glacial acetic acid, and 2 μL of methylene blue.
24. When stacking gel polymerization is complete, remove the comb. Drain the wells completely, using a paper tissue as wick, to remove residual unpolymerized gel solution. At comb and spacer surfaces, gel polymerization is typically incomplete. The high urea concentration of unpolymerized gel solution interferes with the tight application of samples.
25. Remove the bottom spacer from the bottom of the gel assembly and use it to remove any residual Vaseline from the lower surface of the gel.
26. Clamp the gel assembly into the electrophoresis apparatus and fill the lower buffer reservoir with electrophoresis buffer.
27. Use a 5-mL syringe with a bent syringe needle to displace any air bubbles from the bottom of the gel.
28. Samples are applied deep into individual sample wells using a Hamilton microsyringe (rinsed with water between samples) (*see* **Note 12**). For the combination of comb and gel dimensions listed, 50-μL sample will reach a height of 1 cm (*see* **Note 8**). Samples can also be applied to sample wells by any micropipetter with plastic disposable tip. Pipet each sample solution against the long glass plate and let it run to the bottom of the well.

29. Apply reference samples in the outer lanes, which frequently show a slight loss of resolution due to edge effects. The threefold difference in reference protein amounts facilitates correct orientation of the gel following staining and destaining and obviates the need for additional markings. Optionally, apply 50 µL of acidified sample buffer to unused lanes.
30. Gently overlayer the samples with electrophoresis buffer, dispensed from a 5-mL syringe fitted with a 21-gauge needle until all wells are full.
31. Fill the upper buffer reservoir with electrophoresis buffer.
32. Attach the electrical leads between power supply and electrophoresis system: the + lead to the upper and the – lead to the lower reservoir. Note that this is opposite to the SDS gel electrophoresis configuration. Remember, basic proteins such as histones are positively charged and will move toward the cathode (negative electrode).
33. Long (30-cm) gels require 15–20 h of electrophoresis at 300 V in constant voltage mode. They are most easily run overnight. For maximum resolution and stacking capacity, the initial current through a 1-mm thick and 18-cm-wide gel should not exceed 25 mA. Gel electrophoresis is completed in the shortest amount of time in constant power mode with limits of 300 V, 25 mA, and 5 W. The current will drop towards completion of electrophoresis to 6 mA at 300 V.

    Short (15-cm) gels are run at 250 V in constant voltage mode with a similar maximal current, or in constant power mode starting at 25 mA. In the latter example, electrophoresis starts at 25 mA and 135 V, and is complete in 5.5 h at 13 mA and 290 V (*see* **Note 13**).
34. Electrophoresis is complete just before the methylene blue dye exits the gel. Obviously, electrophoresis may be terminated if lesser band resolution is acceptable, or may be prolonged to enhance separation of basic proteins with low gel mobilities, for example, histone H1 variants or phosphorylated forms of histone H1.
35. Open the glass–gel sandwich, and place the separating gel into staining solution, which is gently agitated continuously overnight on a shaker (*see* **Note 14**).
36. Decant the staining solution. The gel can be given a very short rinse in water to remove all residual staining solution.
37. Place the gel in ample destaining solution (*see* **Note 15**). Diffusion of unbound Coomassie dye from the gel is facilitated by the addition of polyurethane foam as an absorbent for free Coomassie dye. To avoid overdestaining and potential loss of protein from the gel (*see* **Note 14**), destaining aids in limited amounts are added to only the first and second destaining solutions. Final destaining is done in the absence of any destaining aids.
38. Record the protein pattern of the gel on film or on a flatbed digital scanner (*see* **Note 16**), possibly with quantitative densitometry *(12)*. Subsequently the gel may be discarded, dried, eluted, blotted *(6)*, or prepared for autoradiography or fluorography as required.

4. Notes

1. Owing to absorbance of the light that initiates gel polymerization, gels thicker than 1.5 mm tend to polymerize better near the light source and produce protein bands that are not perpendicular to the gel surface. For very thick gels, two high-intensity light boxes, placed at either side of the gel assembly, may be required for optimal gel polymerization and resolution.
2. All acrylamide and *bis*-acrylamide solutions are potent neurotoxins and should be dispensed by mechanical pipetting devices.
3. Storage of urea solutions at −20°C minimizes creation of ionic contaminants such as cyanate. The mixed-bed resin ensures that any ions formed are removed. Care should be taken to exclude resin beads from solution taken, for example, by filtration through Whatman no. 1 paper.
4. The stacking ions between which the positively charged proteins and peptides are compressed within the stacking gel during the initial phase of gel electrophoresis are NH_4^+ within the gel compartment and glycine$^+$ in the electrophoresis buffer. Chloride ions interfere with the discontinuous stacking system (*see* **Note 7**). This requires that protein samples should (preferably) be free of chloride salts, and that glycine base rather than glycine salt should be used in the electrophoresis buffer.
5. Amido Black is an alternate staining dye which stains less intensely and destains much slower than Coomassie, but is the better stain for peptides shorter than 30–50 residues.
6. The separating and stacking gels contain 15 vs 4% acrylamide and 0.1 vs 0.16% *N,N′* -methylene (*bis*-acrylamide), respectively, in 1 *M* acetic acid, 0.5% TEMED, 50 m*M* NH_4OH, 8 *M* urea, and 0.0004 % riboflavin-5′-phosphate.

   We have observed that 8 M urea produces the highest resolution of histones in these gels when Triton X-100 is present (*see* Chapter 28). Equal or superior resolution of basic proteins has been reported for AU gels when the urea concentration is reduced to 5 *M*.
7. Acrylamide is photopolymerized with riboflavin or riboflavin 5 -phosphate as initiator, because the ions generated by ammonium persulfate initiated gel polymerization, as used for SDS polyacrylamide gels, interfere with stacking (*see* **Note 4**).
8. The height of stacking gel below the comb determines the volume of samples that can be applied and fully stacked before destacking at the surface of the separating gel occurs. In our experience, 2.5-cm stacking gel height suffices for samples that almost completely fill equally long sample wells. In general, the single blue line of completely stacked proteins and methylene blue dye should be established 1 cm above the separating gel surface. Thus, a 1–1.5 cm stacking gel height will suffice for small-volume samples.
9. Salt-free samples are routinely prepared by exhaustive dialysis against 2.5% (v/v) acetic acid in 3500 molecular weight cutoff dialysis membranes,

followed by freezing at −70°C and lyophilization in conical polypropylene tubes (1.5-, 15-, or 50-mL, filled up to half of nominal capacity) with caps punctured by 21-gauge needle stabs. This method gives essentially quantitative recovery of histones, even if very dilute.

Alternatively, basic proteins can be precipitated with trichloroacetic acid or acetone, acidified by hydrochloric acid to 0.02 *N*, provided that excess salt and acidity is removed by multiple washes with acetone.

Solutions of basic proteins can be used directly, provided that the solution is free of salts that interfere with gel electrophoresis (*see* **Note 11**) and that the concentration of protein is high enough to compensate for the 1.8-fold dilution that occurs during sample preparation. Add 480 mg of urea, 0.05 mL of phenolphthalein, 0.05 mL of concentrated ammonium hydroxide, and 0.05 mL of 1 *M* DTT (freshly prepared) per milliliter sample. If not pink, add more ammonium hydroxide. Leave for 5 min at room temperature. Add 0.05 mL of glacial acetic acid. Measure an aliquot for one gel lane and continue at **step 22**.

10. The amount of protein that can be analyzed in one gel lane depends highly on the complexity of the protein composition. As a guideline, 5–50 μg of total calf thymus histones with five major proteins (modified to varying extent) represents the range between very lightly to heavily Coomassie-stained individual protein bands in 1-mm-thick, 30-cm-long gels using 5-mm-wide comb teeth.
11. Methylene blue is a single blue dye that remains in the gel discontinuity stack of 15% acrylamide separating gels (*see* **Note 4**). Methyl green is an alternate dye marker that contains methylene blue together with yellow and green dye components that remain together in discontinuous mode but that in continuous gel electrophoresis show progressively slower gel mobilities (*see* **Note 17**).
12. As an alternative to **step 28,** electrophoresis buffer is added to the upper buffer reservoir, and all sample wells are filled. Samples are layered under the buffer when dispensed by a Hamilton microsyringe near the bottom of each well. The standard procedure tends to prevent mixing of sample with buffer and thus retains all potential stacking capability.
13. Long gels used in overnight electrophoresis are made on the day that electrophoresis is started. The time for preparation of short gels may prevent electrophoresis on the same day. The nature of the stacking system of the gel (*see* **Note 4**) allows one to prepare a gel on day 1, to store it at room temperature overnight and to initiate electrophoresis on the morning of day 2. To prevent precipitation of urea, gels should not be stored in a refrigerator. The gels should not be stored under electrophoresis buffer as glycine would start to diffuse into the gel and destroy the stacking capability of the system. We routinely store short gels overnight once polymerization is complete

(*see* **step 15**) and before bottom spacer and comb are removed. Saran Wrap® is used to prevent exposed gel surfaces from drying.

14. Be warned that small and strongly basic proteins such as histones are not fixed effectively inside 15% acrylamide gels in methanol–acetic acid without Coomassie. Comparison of identical gels, one fixed and stained as described and the other placed first in destain solution alone for several hours, followed by regular staining by Coomassie, reveals that 90% or more of core histones are lost from the gel. We speculate that the Coomassie dye helps to retain histones within the gel matrix. This is consistent with the observation that gradual loss of Coomassie intensity of histone bands is observed upon exhaustive removal of soluble dye.
15. Note that the gel increases significantly in size when transferred from the gel plates into the staining solution. The compositions of staining and detaining solvent mixtures are identical and changes in gel size are not observed upon destaining.
16. A standard method to record the protein staining pattern in Coomassie-stained polyacrylamide gels has been Polaroid photography, using an orange filter to increase contrast, with the gel placed on the fluorescent light box, covered by a glass plate to prevent Coomassie staining of the typical plastic surface. Polaroid negatives can be scanned but suffer from a nonlinear response of density, even within the range in which careful Coomassie staining leads to near-linear intensity of protein band staining.

    With the advent of 24+ bit color flatbed scanners with transmitted light capabilities and linear density capabilities in excess of three optical densities, it has become easy to record, and quantitate, intensity of protein staining patterns. Care should be taken to develop a standard scanning setup, using the full dynamic range (typically all three colors used at their full range, 0–255 for a 24-bit scanner, excluding automatic adjustments for density and contrast). A standard gamma correction value should be determined, using an optical density wedge (Kodak), to ensure that the density response is linear. Placing a detained polyacrylamide gel on the gel scanner, one should cover the top of the gel with an acetate film to prevent surface reflection abnormalities and prevent touching of the transilluminating light source surface to the film, avoiding moiré interference patterns. Recording gel patterns at 300 dpi in full color and saving loss-less tiff files facilitates faithful replication of experimental results (**Fig. 1**). Quantitative densitometry programs can use the image files.
17. Gel preelectrophoresis until the methylene blue dye, and thus all ammonium ions (*see* **Note 4**), have exited the separating gel converts this gel system into a continuous one. This option can be used to separate small proteins and peptides that do not destack at the boundary with the separating gel. Although this option is available, one should consider alternatives,

such as increasing the acrylamide concentration of the separating gel. West and co-workers have developed a system with 40–50% polyacrylamide gels that is similar to the one described here and that has been optimized for the separation of small basic peptides ***(13)***.

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28

Acid–Urea–Triton Polyacrylamide Gel Electrophoresis of Histones

Jakob H. Waterborg

1. Introduction

Acid-urea polyacrylamide gels are capable of separating basic histone proteins provided they differ sufficiently in size and/or effective charge (*see* Chapter 27). Separation between similarly sized and charged molecules, such as the histones H2A, H2B, and the H3 forms of most organisms, can typically not be achieved. Zweidler discovered that core histones but not linker histones (*see* **Note 1**) bind the nonionic detergent Triton ***(1)***. Generally, Triton is added to an acetic acid-urea (AU) gel system to separate core histone sequence variants and histone species with overlapping AU gel patterns. This type of gel is known as an AUT or a TAU gel. To date, a single example is known where addition of Triton X-100 has allowed separation of a nonhistone primary sequence variation, the hydrophobic replacement variant of phenylalanine by leucine in fetal hemoglobin ***(2)***.

The binding of Triton to a core histone increases the effective mass of the protein within the gel without affecting its charge, and thus reduces its mobility during electrophoresis. Separation between most or all core histone proteins of diverse species can virtually always be obtained by adjusting concentrations of Triton and of urea, which appears to act as a counteracting, dissociating agent ***(3)***. Experimentally, an optimal balance can be determined by gradient gel electrophoresis with a gradient of urea ***(4)*** or Triton ***(5)***. The Triton gradient protocol in the discontinuous gel system, developed by Bonner and co-workers ***(6)***, is described in **Subheading 3**. It has a distinct advantage over the urea gradient protocol. Generally, it can identify a core histone protein band as belonging to histone H4, H2B, H3, or H2A. In this order the apparent affinities for Triton X-100 increase sharply ***(5,7,8)***. An example of such a separation of a crude

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mixture of histones with nonhistone proteins from a tobacco callus culture is shown in **Fig. 1A**. In addition, a detailed working protocol for a long AUT gel at 9 m*M* Triton and 8 *M* urea is provided. It describes the protocol used extensively in my laboratory for the analysis of core histones, especially of histone H3, in dicot *(7)* and monocot plants *(8)*, in the green alga *Chlamydomonas* *(9)* and in budding yeast *Saccharomyces cerevisiae* *(10)*. **Figure 1B** shows an example of the differentially acetylated histone H3 variant proteins of tobacco, purified by reversed-phase high performance liquid chromatography (HPLC) *(7)*. The protocol description parallels directly the acid-urea gel protocol described in Chapter 27 which also provides details for the use of different gel dimensions.

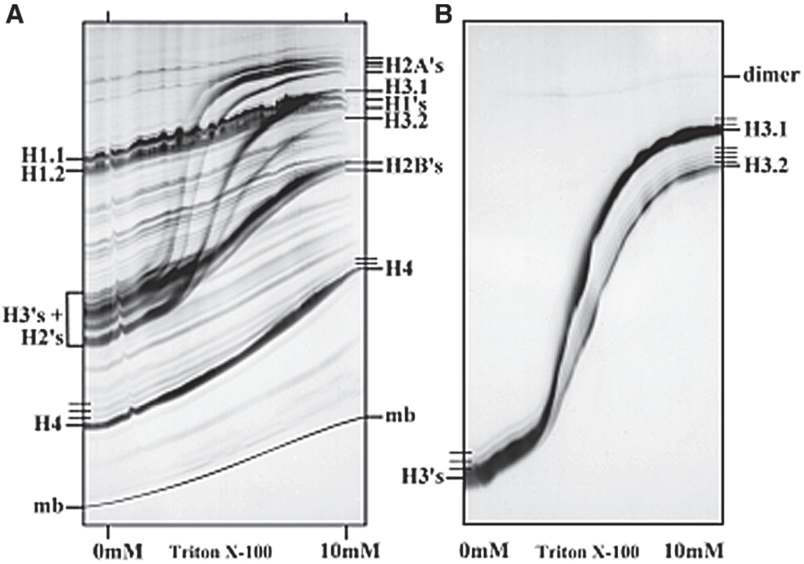

Fig. 1. Acid-urea gradient gel electrophoresis of tobacco histones. **(A)** A crude preparation of basic proteins, extracted from callus cultures of tobacco *(7)*, was electrophoresed on the gradient AUT gel and stained with Coomassie, as described. Marks at top and bottom indicate the joints between the gel compartments, from left to right, 0 m*M* Triton, 0–10 m*M* Triton gradient, and 10 m*M* Triton. The buffer front is marked by the methylene blue dye (mb). The identified histone bands are marked: two variants for histone H1, histone H4 with detectable mono- through triacetylation, two histone H2B variants, two histone H3 variants (H3.1 and the more highly acetylated histone H3.2) and, at least four histone H2A variant forms. Note that the Triton affinity of the core histones increases in the typical order of H4, H2B, H3, and H_2A. **(B)** Tobacco histone H3, purified by reversed-phase HPLC as a mixture of low acetylated histone H3.1 and highly acetylated histone H3.2, on a gradient AUT gel. The 0 and 10 m*M* Triton concentrations coincide with the edge of the figure. The presence of a small amount of histone H3 dimer is indicated.

2. Materials

1. Vertical gel apparatus for long (30-cm) slab gels. A gel electrophoresis apparatus that allows gel polymerization between the glass plates with spacers, without being assembled in the apparatus, is required. This allows the even and complete photopolymerization of the acrylamide gel compartments in both orientations used. In this type of apparatus the glass-gel sandwich is typically clamped to the lower buffer reservoir, which acts as a stand, after which the upper buffer reservoir is clamped to the top of the gel assembly. Details of the procedure are described for a fairly standard and flexible gel apparatus that uses two rectangular glass plates (4-mm-thick standard plate glass with sanded edges), 21 cm wide and 32.5 and 35.5 cm long, respectively. The Plexiglas bottom buffer reservoir with platinum electrode is 22.5 wide with three sides 5 cm high and one of the long sides 12.5 cm high. To this side the glass plates are clamped. The upper buffer reservoir with platinum electrode and with a similar buffer capacity is 18 cm wide with one long side enlarged to measure 21 cm wide by 10 cm high. It contains a cutout of 18 cm wide and 3.5 cm high that allows access of the upper reservoir buffer to the top of the gel. The 21-cm wide Plexiglas plate is masked with 5-mm-thick closed-cell neoprene tape (weather strip), and provides a clamping ridge for attachment to the top of the glass–gel sandwich.
2. Spacers and combs are cut from 1 mm Teflon sheeting. Required are two side spacers (1.5 × 35 cm), one bottom spacer (0.5 × 24 cm) and, for the gradient gel, one temporary spacer (1.5 × 21 cm). Added to the top of the side spacers is 3-cm adhesive, closed-cell neoprene tape (weather strip, 14 mm wide and 5 mm thick). A 15-cm-wide comb with 20 teeth of 5 × 30 mm, cut from a rectangle of 15 × 5 cm Teflon, is used with the teeth pointing down for the regular gel and with the teeth pointing up as a block comb for the gradient gel.
3. Vaseline pure petroleum jelly.
4. Acrylamide stock solution: 60% (w/v) acrylamide, highest quality available, in water (*see* **Note 2**). The acrylamide is dissolved by stirring. Application of heat should be avoided, if possible, to prevent generating acrylic acid. The solution can be kept at least for 3 mo on the laboratory shelf at room temperature. Storage at 4°C can exceed 2 yr without detectable effects.
5. *N*,*N′* -Methylene *bis*-acrylamide stock solution: 2.5% (w/v) in water (*see* **Note 2**).
6. Glacial acetic acid (HAc): 17.5 *M*.
7. Concentrated ammonium hydroxide: NH_4OH, 28–30%, approx 15 *M*.
8. Triton X-100 stock solution: 25% (w/v) in water (0.4 *M*) is used, as it is much easier to dispense accurately than 100%.
9. *N*,*N*,*N′*, *N′* -Tetramethylenediamine (TEMED), stored at 4°C.
10. Riboflavin 5′-phosphate solutions: 0.006% (R5P) and 0.06% (R5P-hi) (w/v) in water. The lower concentration solution is used for the regular gel and for all stacking gels. It is stable for more than 6 mo if kept dark and stored at 4°C. Riboflavin 5′ -phosphate readily dissolves at the higher concentration, which is used for the gradient gel formulation, but it cannot be stored for more than 1 d at room temperature in the dark, as precipitates form readily upon storage at 4°C.

11. Glycerol.
12. Urea, ultrapure quality.
13. Side-arm suction flasks with stoppers, magnetic stirrer, stirrer bar, and water-aspirator vacuum. Measuring cylinders with silicon-rubber stoppers. Pipets and pipetting bulbs or mechanical pipetting aids. Plastic syringes, 1 and 5 mL, with 20-gauge needles.
14. Fluorescent light box with diffuser for even light output and with the possibility to stand vertically. Light intensity should equal or exceed 5 klx at a distance of 5–10 cm. A high-quality X-ray viewing light box with three 40-W bulbs typically will meet this specification.
15. Aluminum foil.
16. Gradient mixer to prepare a linear concentration gradient with a volume of 30 mL. We use with success the 50-mL Jule gradient maker (Research Products International, Mt. Prospect, IL) with two 25-mL reservoirs.
17. Electrophoresis power supply with constant voltage mode at 300–500 V with up to 50 mA current, preferably with a constant power mode option.
18. Urea stock solution: 8 *M* urea in water. An aliquot of 40 mL with 1 g of mixed-bed resin (Bio-Rad [Hercules, CA] AG 501-X8) can be used repeatedly over a period of months if refrozen and stored between uses at −20°C (*see* **Note 3**).
19. Phenolphthalein indicator solution: 1% (w/v) in 95% ethanol, stored indefinitely at room temperature in a closed tube.
20. Dithiothreitol (DTT, Cleland's reagent) is stored at 4°C and is weighed freshly for each use.
21. Methylene blue running front indicator dye solution: 2% (w/v) in sample buffer (*see* **Subheading 3.**, **step 16**).
22. Reference histones: Total calf thymus histones (Worthington, Freehold, NJ), stored dry at 4°C indefinitely or in solution at −80°C in 50-µL aliquots of 5 mg/mL in water for more than 1 yr (*see* **Subheading 3.**, **step 17**).
23. Glass Hamilton microsyringe (100 µL) with Teflon-tipped plunger.
24. Electrophoresis buffer: 1 *M* acetic acid, 0.1 *M* glycine (*see* **Note 4**). This solution can be made in bulk and stored indefinitely at room temperature.
25. Destaining solution: 20% (v/v) methanol, 7% (v/v) acetic acid in water.
26. Staining solution: Dissolve a fresh 0.5 g of Coomassie Brilliant Blue R250 in 500 mL of destaining solution. If the dye dissolves incompletely, the solution should be filtered through Whatman no. 1 paper to prevent staining artifacts.
27. Glass tray for gel staining and destaining.
28. Rotary or alternating table top shaker.
29. Destaining aid: Polyurethane foam.

3. Method

1. For the AUT regular gel, follow **steps a–i** and continue at **step 3**.
    a. Assemble a sandwich of two clean glass plates with two side spacers and a bottom spacer, lightly greased with Vaseline to obtain a good seal, clamped along all sides with 2-in binder clamps. The triangular shape of these clamps facilitates the vertical, freestanding position of the gel assembly a few centimeters in front of the vertical light box (**Fig. 2A**).

   b. Separating gel solution: pipet into a 100-mL measuring cylinder 17.5 mL of acrylamide stock solution, 2.8 mL of *bis*-acrylamide stock solution, 4.2 mL of glacial acetic acid, and 0.23 mL of concentrated ammonium hydroxide solutions (*see* **Note 5**).
   c. Add 33.6 g of urea and add distilled water to a total volume of 63.5 mL.
   d. Stopper the measuring cylinder and place on a rotary mixer until all urea has dissolved. Add water to 63.5 mL, if necessary.
   e. Transfer this solution to a 200-mL sidearm flask with magnetic stir bar on a magnetic stirrer. While stirring vigorously, stopper the flask and apply water-aspirator vacuum. Initially, a cloud of small bubbles of dissolved gas arises, which clears after just a few seconds. Terminate vacuum immediately to prevent excessive loss of ammonia.
   f. Add 1.575 mL of Triton, 0.35 mL of TEMED and 4.67 mL of R5P (*see* **Note 6**), mix, and pipet immediately between the glass plates to a marking line 5 cm below the top of the shorter plate (*see* **Note 7**).
   g. Carefully apply 1 mL of distilled water from a 1-mL syringe with needle along one of the glass plates to the top of the separating gel solution to obtain a flat separation surface.
   h. Switch the light box on and place a reflective layer of aluminum foil behind the gel to increase light intensity and homogeneity. Gel polymerization becomes detectable within 2 min and is complete in 15–30 min.
   i. Switch the light box off, completely drain the water from between the plates, and insert the comb 2.5 cm between the glass plates. The tops of the teeth should always remain above the top of the short glass plate.
2. For the Triton gradient gel, follow **steps a–q** and continue at **step 3**.
   a. Assemble a sandwich of two clean glass plates with one side spacer, a bottom spacer, and a temporary spacer, clamped with large binder clamps (Fig. 2B). The spacers are lightly greased with Vaseline to obtain a good seal but the amount of grease on the temporary spacer should be as low as possible. No grease should be present on the side of this spacer facing the buffer compartment to assure a flawless destacking surface. Place magic marker guidance lines at 4, 14, and 18 cm above the long side spacer (Fig. 2B). Place the assembly horizontally a few centimeters in front of the vertical light box and add a reflective layer of aluminum behind the gel.
   b. Heavy gel separating gel solution: Pipet into a 50-mL graduated, capped polypropylene tube, 4.0 mL of glycerol, 10 mL of acrylamide stock solution, 1.6 mL of bis-acrylamide stock solution, 2.4 mL of glacial acetic acid, and 0.13 mL of concentrated ammonium hydroxide. Add 19.2 g of urea. Add distilled water to a total volume of 38.5 mL (see Note 5).
   c. Light gel separating gel solution: Pipet into a 50-mL graduated, capped polypropylene tube, 10 mL of acrylamide stock solution, 1.6 mL of *bis*-acrylamide stock solution, 2.4 mL of glacial acetic acid, and 0.13 mL of concentrated ammonium hydroxide. Add 19.2 g of urea. Add distilled water to a total volume of 39.5 mL.

   d. Place the closed tubes on a rotary mixer until all urea has dissolved. Add water to the required final volume, if necessary.
   e. Transfer each solution to a 100-mL sidearm flask with magnetic stir bar on a magnetic stirrer. While stirring vigorously, stopper the flask and apply water-aspirator vacuum. Initially, a cloud of small bubbles of dissolved gas arises, which clears after just a few seconds. Terminate vacuum immediately to prevent excessive loss of ammonia.
   f. Return the gel solutions after degassing to the capped tubes.
   g. Add to the heavy gel solution 1.0 mL of Triton stock solution.
   h. Wrap each tube into aluminum foil to protect the gel solution from light (*see* **Note 6**).
   i. Prepare freshly concentrated (0.06%) riboflavin 5′ -phosphate (R5P-hi) solution (*see* **Subheading 2.**, **step 10**).
   j. Add to each gel solution 0.20 mL of TEMED and 0.27 mL of R5P-hi, and mix.
   k. Under conditions of darkness (or very reduced light levels), pipet approx 12 mL of heavy gel solution between the plates, up to the 4-cm heavy gel surface mark (Fig. 2B).
   l. Turn the light box on for 2 min only. This allows the heavy gel to initiate polymerization and gelling, but retains sufficient unpolymerized acrylamide to fuse this gel partition completely into the gel layered on top.
   m. In darkness, set up the gradient maker with 15 mL of heavy gel and 15 mL of light gel solutions and create slowly, over a period of at least several minutes, a linear gradient that flows slowly and carefully between the glass plates. Empirically, we have observed that a 10% increase over the calculated volume of the 27 × 10 × 0.1 cm gradient partition will create a gradient that is complete near the marker line between gradient and light partition (Fig. 2B).
   n. Turn the light box on for 2 min only.
   o. In darkness, slowly add light gel solution on top of the partially polymerized gradient gel until the upper marker line.
   p. Switch the light box on for complete gel polymerization in 15–30 min.
   q. Remove the temporary spacer from the assembly. Insert a slightly greased spacer along the polymerized light gel. Reclamp the assembly. Insert the 20-well comb in the middle and upside down as a block comb for 2.5 cm below the edge of the shorter glass plate. Reposition the assembly vertically between light box and aluminum foil (**Fig. 2A**).
3. Stacking gel solution, made in parallel to **steps 1b–d** or **steps 2b–d**: Pipet into a 25-mL measuring cylinder 1.34 mL of acrylamide stock solution, 1.28 mL of *bis*-acrylamide stock solution, 1.14 mL of glacial acetic acid, and 0.07 mL of concentrated ammonium hydroxide (*see* **Note 5**).
4. Add 9.6 g of urea and add distilled water to a total volume of 18.6 mL.
5. Stopper the measuring cylinder and place on a rotary mixer until all urea has dissolved. Add water to 18.6 mL, if necessary.
6. Once the separating gel has polymerized, transfer the stacking gel solution to a 50-mL sidearm flask with stir bar and degas as in **step 1e**.
7. Add 0.1 mL of TEMED and 1.3 mL of R5P, mix and pipet between the plates between the comb teeth.

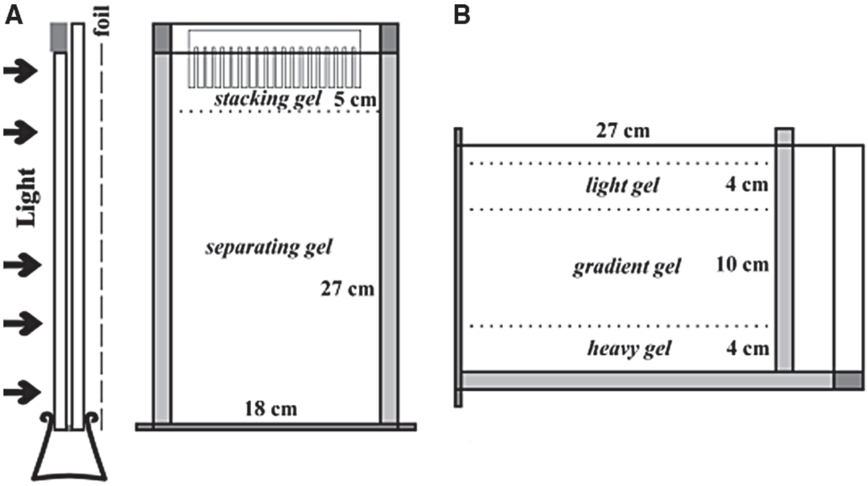

Fig. 2. **(A)** Side and front view of a long AUT gel assembly for 20 samples. The Teflon side spacers are shown in light gray, the bottom Teflon spacer and the neoprene blocks in dark gray. The fluorescent light source and reflecting aluminum foil are shown. One clamp is shown in the side view to demonstrate the independent vertical stand of this assembly and to represent the clamps all around the assembly at all spacer locations. **(B)** Front view of the AUT gradient assembly as used when the separation gel partitions are formed and polymerized.

8. Displace air bubbles, especially in the gradient assembly where residual Vaseline may interfere with gel solution flow. In this case, all (mini) bubbles should be carefully removed from the separation gel surface that will act as the destacking boundary.
9. Switch the light box on and allow complete gel polymerization in 30–60 min.
10. Sample buffer is freshly prepared when the separation gel is polymerizing. The preferred protein sample is a salt-free lyophilisate (*see* **Note 8**). For regular gels, determine the approximate volume of sample buffer required, depending on the number of samples. For a gradient gel 0.5–1.5 mL sample buffer appears optimal.
11. Weigh DTT into a sample buffer preparation tube for a final concentration of 1 *M*, that is, 7.7 mg/mL.
12. Per 7.7 mg of DTT, add 0.9 mL of 8 *M* urea stock solution, 0.05 mL of phenolphthalein, and 0.05 mL of concentrated ammonium hydroxide to the tube to obtain the intensely pink sample buffer.
13. Add 0.05 mL of sample buffer per sample tube with lyophilized protein to be analyzed in one gel lane (*see* **Note 9**). Add 1 mL of sample buffer to sample for one gradient gel. To assure full reduction of all proteins by DTT, the pH must be above 8.0. If the pink phenolphthalein color disappears because of residual acid in the sample, a few microliters of concentrated ammonium hydroxide should be added to reach an alkaline pH.

14. Limit the time for sample solubilization and reduction to 5 min at room temperature to minimize the possibility of protein modification at alkaline pH by reactive urea side reactions, for example, by modification of cysteine residues by cyanate.
15. Acidify the sample by addition of 1/20 volume of glacial acetic acid.
16. Add methylene blue running front dye: 2 µL per gel lane or 50 µL for a gradient gel.
17. For a regular AUT gel, prepare reference histone samples: To 2 and 6 µL reference histone solution with 10 and 30 µg of total calf thymus histones, one adds 40 µL sample buffer (**step 12**), 2.5 µL of glacial acetic acid, and 2 µL of methylene blue.
18. When stacking gel polymerization is complete, remove the comb. Drain the wells completely, using a paper tissue as wick, to remove residual unpolymerized gel solution. At comb and spacer surfaces, gel polymerization is typically incomplete. The high urea concentration of unpolymerized gel solution interferes with the tight application of samples.
19. Remove the bottom spacer from the gel assembly gel assembly and use it to remove any residual Vaseline from the lower surface of the gel.
20. Clamp the gel assembly into the electrophoresis apparatus and fill the lower buffer reservoir with electrophoresis buffer.
21. Use a 5-mL syringe with a bent syringe needle to displace any air bubbles from the bottom of the gel.
22. For regular gel, follow **steps a–c** and continue at **step 24**.
    a. Samples are applied deep into individual sample wells by Hamilton microsyringe (rinsed with water between samples) (*see* **Note 10**). For the combination of comb and gel dimensions listed, a 50-µL sample will reach a height of 1 cm (*see* **Note 7**). Samples can also be applied to sample wells by any micropipetter with plastic disposable tip. Pipet each sample solution against the long glass plate and let it run to the bottom of the well.
    b. Apply reference samples in the outer lanes, which frequently show a slight loss of resolution due to edge effects. The threefold difference in reference protein amounts facilitates correct orientation of the gel following staining and destaining and obviates the need for additional markings. Optionally, apply 50 µL of acidified sample buffer to unused lanes.
    c. Gently overlayer the samples with electrophoresis buffer, dispensed from a 5-mL syringe fitted with a 21-gauge needle until all wells are full.
23. For gradient gel, follow **steps a–c** and continue at **step 24**.
    a. Fill the block well with electrophoresis buffer.
    b. Use a level to confirm that the bottom of the preparative well is exactly horizontal.
    c. Distribute the total sample evenly across the width of the well using a 250-µL Hamilton microsyringe. Limited mixing of sample and electrophoresis buffer will facilitate even loading and is easily dealt with by the strong stacking capability of the gel system (*see* **Note 11**).
24. Fill the upper buffer reservoir with electrophoresis buffer.
25. Attach the electrical leads between power supply and electrophoresis system: the + lead to the upper and the – lead to the lower reservoir. Note that this is opposite to the SDS gel electrophoresis configuration. Remember, basic proteins such as histones are positively charged and will move toward the cathode (negative electrode).

26. Long (30-cm) gels require 15–20 h of electrophoresis at 300 V in constant voltage mode. They are most easily run overnight. For maximum resolution and stacking capacity the initial current through a 1 mm thick and 18 cm wide gel should not exceed 25 mA. Gel electrophoresis is completed in the shortest amount of time in constant power mode with limits of 300 V, 25 mA, and 5 W. The current will drop toward completion of electrophoresis to 6 mA at 300 V.

    Gradient gel electrophoresis typically takes a few more hours. At 300 V constant voltage the electrophoretic current is reduced, in particular at the heavy side of the gel due to the reduced water concentration caused by the inclusion of glycerol. The ion front with methylene blue dye reflects this in a distinct curvature (**Fig. 1A**).
27. Electrophoresis is complete just before the methylene blue dye exits the gel. Obviously, electrophoresis may be terminated earlier if lesser band resolution is acceptable, or may be prolonged to enhance separation of histones with low gel mobilities, for example, histone H3 variants (**Fig. 1B**).
28. Open the glass–gel sandwich and place the separating gel into staining solution, which is gently agitated continuously overnight on a shaker (*see* **Note 12**).
29. Decant the staining solution. The gel can be given a very short rinse in water to remove all residual staining solution.
30. Place the gel in ample destaining solution. Diffusion of unbound Coomassie dye from the gel is facilitated by the addition of polyurethane foam as an absorbent for free Coomassie dye. To avoid overdestaining and potential loss of protein from the gel, polyurethane foam in limited amounts is added to only the first and second destaining solutions. Final destaining is done in the absence of any foam.
31. Record the protein pattern of the gel on film (**Fig. 1**) or on a flatbed digital scanner (*see* **Note 13**). Subsequently the gel may be discarded, dried, or prepared for autoradiography or fluorography as required. Blotting of AUT gels requires removal of Triton to allow successful histone transfer (*see* **Note 14**).

4. Notes

1. Core histones share a 65-basepair helix-turn-helix-turn-helix histone-fold motif which is the basis of the hydrophobic pairwise interaction between histones H2A and H2B and between histones H3 and H4. Our studies of histone H3 variant proteins from HeLa, *Physarum* ***(11)***, Chlamydomonas ***(9)*** and several plant species ***(5, 7, 8)*** support the notion that the characteristic differences in Triton X-100 affinity—Triton affinity: H2A > H3 > H2B > H4 (**Fig. 1**)—depend on sequence differences at residues whose side chains are mapped to the inside of the histone fold ***(12, 13)***. To date, many proteins involved in DNA organization and transcription have been shown to share the histone fold, creating homo- and hetero-dimer complexes ***(14)***. However, none have yet been analyzed by AUT gradient gel electrophoresis to confirm the predicted interaction with Triton X-100.
2. All acrylamide and *bis*-acrylamide solutions are potent neurotoxins and should be dispensed by mechanical pipetting devices.

3. Storage of urea solutions at −20°C minimizes creation of ionic contaminants such as cyanate. The mixed-bed resin assures that any ions formed are removed. Care should be taken to exclude resin beads from solution taken, for example, by filtration through Whatman no. 1 paper.
4. The stacking ions between which the positively charged proteins and peptides are compressed within the stacking gel during the initial phase of gel electrophoresis are NH_4^+ within the gel compartment and glycine$^+$ in the electrophoresis buffer. Chloride ions interfere with the discontinuous stacking system. This requires that protein samples should (preferably) be free of chloride salts, and that glycine base rather than glycine salt should be used in the electrophoresis buffer.
5. The separating and stacking gels contain 15 vs 4% acrylamide and 0.1 vs 0.16% *N*,*N*′-methylene *bis*-acrylamide, respectively, in 1 *M* acetic acid, 0.5% TEMED, 50 m*M* NH_4OH, 8 *M* urea, and 0.0004% riboflavin 5 -phosphate. The concentration of Triton X-100 in the separating gel of the regular system is 9 m*M*, optimal for the separation desired in our research for histone H3 variant forms of plants and algae.

   In the gradient system, glycerol and Triton concentrations change in parallel: 10% (v/v) glycerol and 10 m*M* Triton in the heavy gel (**Fig. 2B**) and a gradient between 10 and 0% (v/v) glycerol and between 10 and 0 m*M* Triton in the gradient from heavy to light (**Fig. 2B**).
6. Acrylamide is photopolymerized with riboflavin or riboflavin 5′ -phosphate as initiator because the ions generated by ammonium persulfate initiated gel polymerization, as used for SDS polyacrylamide gels, interfere with stacking (*see* **Note 4**).
7. The height of stacking gel below the comb determines the volume of samples that can be applied and fully stacked before destacking at the surface of the separating gel occurs. In our experience, a 2.5 cm stacking gel height suffices for samples that almost completely fill equally long sample wells. In general, the single blue line of completely stacked proteins and methylene blue dye should be established 1 cm above the separating gel surface. Thus, 1–1.5 cm stacking gel height will suffice for small volume samples.
8. Salt-free samples are routinely prepared by exhaustive dialysis against 2.5% (v/v) acetic acid in 3500 mol wt cutoff dialysis membranes, followed by freezing at −70°C and lyophilization in conical polypropylene tubes (1.5-, 15-, or 50-mL, filled up to half of nominal capacity) with caps punctured by 21-gauge needle stabs. This method gives essentially quantitative recovery of histones, even if very dilute. (For alternative methods, *see* Chapter 27).
9. The amount of protein that can be analyzed in one gel lane depends highly on the complexity of the protein composition. As a guideline, 5–50 µg of total calf thymus histones with five major proteins (modified to varying extent) represents the range between very lightly to heavily

Coomassie-stained individual protein bands in 1-mm-thick, 30-cm-long gels using 5-mm-wide comb teeth.

The optimal amount of protein for a gradient gel also depends on the number of protein species that must be analyzed. In general, a gradient using the block comb used, equivalent to the width of 30 gel lanes, should be loaded with 30 times the sample for one lane.

10. As an alternative to **step 22**, electrophoresis buffer is added to the upper buffer reservoir and all sample wells are filled. Samples are layered under the buffer when dispensed by a Hamilton microsyringe near the bottom of each well.
11. The preparative well of a gradient gel should not be loaded with sample prior to the addition of electrophoresis buffer. Uneven distribution of sample cannot be avoided when buffer is added.
12. For unknown reasons, the polyacrylamide gel below the buffer front tends to stick tightly to the glass in an almost crystalline fashion. This may cause tearing of a gradient gel at the heavy gel side below the buffer front. Since attachment to one of the gel plates is typically much stronger, one can release the gel without problems by immersing the glass plate, with gel attached, in the staining solution.
13. A standard method to record the protein staining pattern in Coomassie-stained polyacrylamide gels has been Polaroid photography, using an orange filter to increase contrast, with the gel placed on the fluorescent light box, covered by a glass plate to prevent Coomassie staining of the typical plastic surface. Polaroid negatives can be scanned but suffer from a nonlinear response of density, even within the range where careful Coomassie staining leads to near-linear intensity of protein band staining.

    With the advent of 24+ bit color flatbed scanners with transmitted light capabilities and linear density capabilities in excess of three optical densities, it has become easy to record, and quantitate, intensity of protein staining patterns. Care should be taken to develop a standard scanning setup, using the full dynamic range (typically all three colors used at their full range, 0–255 for a 24-bit scanner; excluding automatic adjustments for density and contrast). A standard gamma correction value should be determined, using an optical density wedge (Kodak), to assure that the density response is linear. Placing a destained polyacrylamide gel on the gel scanner, one should cover the top of the gel with an acetate film to prevent surface reflection abnormalities and prevent touching of the transilluminating light source surface to the film, avoiding moiré interference patterns. Recording gel patterns at 300 dpi in full color and saving loss-less tiff files facilitates faithful replication of experimental results (**Fig. 1**). Quantitative densitometry programs can use the image files.
14. Triton X-100 interferes with native-mode electrotransfer of histones to nitrocellulose ***(15)***. Exchange of Triton by SDS under acidic conditions allows Western blotting ***(16)*** (*see* Chapter 61).

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29

Isoelectric Focusing of Proteins in Ultra-Thin Polyacrylamide Gels

John M. Walker

1. Introduction

Isoelectric focusing (IEF) is an electrophoretic method for the separation of proteins, according to their isoelectric points (pI), in a stabilized pH gradient. The method involves casting a layer of support media (usually a polyacrylamide gel but agarose can also be used) containing a mixture of carrier ampholytes (low-mol-wt synthetic polyamino-polycarboxylic acids). When using a polyacrylamide gel, a low percentage gel (4%) is used since this has a large pore size, which thus allows proteins to move freely under the applied electrical field without hindrance. When an electric field is applied across such a gel, the carrier ampholytes arrange themselves in order of increasing pI from the anode to the cathode. Each carrier ampholyte maintains a local pH corresponding to its pI and thus a uniform pH gradient is created across the gel. If a protein sample is applied to the surface of the gel, where it will diffuse into the gel, it will also migrate under the influence of the electric field until it reaches the region of the gradient where the pH corresponds to its isoelectric point. At this pH, the protein will have no net charge and will therefore become stationary at this point. Should the protein diffuse slightly toward the anode from this point, it will gain a weak positive charge and migrate back towards the cathode, to its position of zero charge. Similarly diffusion toward the cathode results in a weak negative charge that will direct the protein back to the same position. The protein is therefore trapped or "focused" at the pH value where it has zero overall charge. Proteins are therefore separated according to their charge, and not size as with SDS gel electrophoresis. In practice the protein samples are loaded onto the gel before the pH gradient is formed. When a voltage difference is applied, protein migration and pH gradient formation occur simultaneously.

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Traditionally, 1–2 mm thick isoelectric focusing gels have been used by research workers, but the relatively high cost of ampholytes makes this a fairly expensive procedure if a number of gels are to be run. However, the introduction of thin-layer isoelectric focusing (where gels of only 0.15 mm thickness are prepared, using a layer of electrical insulation tape as the "spacer" between the gel plate) considerably reduced the cost of preparing IEF gels, and such gels are therefore described in this chapter. Additional advantages of the ultra-thin gels over the thicker traditional gels are the need for less material for analysis, and much quicker staining and destaining times. Also, a permanent record can be obtained by leaving the gel to dry in the air, i.e., there is no need for complex gel-drying facilities. The tremendous resolution obtained with IEF can be further enhanced by combinations with SDS gel electrophoresis in the form of 2D gel electrophoresis.

2. Materials

1. Stock acrylamide solution: acrylamide (3.88 g), bis-acrylamide (0.12 g), sucrose (10.0 g). Dissolve these components in 80 mL of water. This solution may be prepared some days before being required and stored at 4°C (*see* **Note 1**).
2. Riboflavin solution: This should be made fresh, as required. Stir 10 mg riboflavin in 100 mL water for 20 min. Stand to allow undissolved material to settle out (or briefly centrifuge) (*see* **Note 2**).
3. Ampholytes: pH range 3.5–9.5 (*see* **Note 3**).
4. Electrode wicks: 22 cm × 0.6 cm strips of Whatman No. 17 filter paper.
5. Sample loading strips: 0.5 cm square pieces of Whatman No. 1 filter paper, or similar.
6. Anolyte: 1.0 *M* H_3PO_4.
7. Catholyte: 1.0 *M* NaOH.
8. Fixing solution: Mix 150 mL of methanol and 350 mL of distilled water. Add 17.5 g sulfosalicylic acid and 57.5 g trichloroacetic acid.
9. Protein stain: 0.1% Coomassie brilliant blue R250 in 50% methanol, 10% glacial acetic acid. N.B. Dissolve the stain in the methanol component first.
10. Glass plates: 22 cm × 12 cm. These should preferably be of 1 mm glass (to facilitate cooling), but 2 mm glass will suffice.
11. PVC electric insulation tape: The thickness of this tape should be about 0.15 mm and can be checked with a micrometer. The tape we use is actually 0.135 mm.
12. A bright light source.

3. Methods

1. Thoroughly clean the surfaces of two glass plates, first with detergent and then methylated spirit. It is *essential* for this method that the glass plates are clean.
2. To prepare the gel mold, stick strips of insulation tape, 0.5 cm wide, along the four edges of one glass plate. Do not overlap the tape at any stage. Small gaps at the

join are acceptable, but do *not* overlap the tape at corners as this will effectively double the thickness of the spacer at this point.

3. To prepare the gel solution, mix the following: 9.0 mL acrylamide, 0.4 mL ampholyte solution, and 60.0 µL riboflavin solution.

   N.B. Since acrylamide monomer is believed to be neurotoxic, **steps 4–6** must be carried out wearing protective gloves.
4. Place the glass mold in a spillage tray and transfer ALL the gel solution with a Pasteur pipet along one of the short edges of the glass mold. The gel solution will be seen to spread slowly toward the middle of the plate.
5. Take the second glass plate and place one of its short edges on the taped edge of the mold, adjacent to the gel solution. Gradually lower the top plate and allow the solution to spread across the mold. Take care not to trap any air bubbles. If this happens, carefully raise the top plate to remove the bubble and lower it again.
6. When the two plates are together, press the edges firmly together (NOT the middle) and discard the excess acrylamide solution spilled in the tray. Place clips around the edges of the plate and thoroughly clean the plate to remove excess acrylamide solution using a wet tissue.
7 Place the gel mold on a light box (*see* **Note 4**) and leave for at least 3 h to allow polymerization (*see* **Note 5**). Gel molds may be stacked at least three deep on the light box during polymerization. Polymerized gels may then be stored at 4°C for at least 2 mo, or used immediately. If plates are to be used immediately they should be placed at 4°C for 15 min, since this makes the separation of the plates easier.
8. Place the gel mold on the bench and remove the top glass plate by inserting a scalpel blade between the two plates and *slowly* twisting to remove the top plate. (N.B. Protect eyes at this stage.) The gel will normally be stuck to the side that contains the insulation tape. Do *not* remove the tape. Adhesion of the gel to the tape helps fix the gel to the plate and prevents the gel coming off in the staining/destaining steps (*see* **Note 6**).
9. Carefully clean the underneath of the gel plate and place it on the cooling plate of the electrophoresis tank. Cooling water at 10°C should be passing through the cooling plate.
10. Down the full length of one of the longer sides of the gel lay electrode wicks, uniformly saturated with either 1.0 *M* phosphoric acid (anode) or 1.0 *M* NaOH (cathode) (*see* **Note 7**).
11. Samples are loaded by laying filter paper squares (Whatman No. 1, 0.5 cm × 0.5 cm), wetted with the protein sample, onto the gel surface. Leave 0.5 cm gaps between each sample. The filter papers are prewetted with 5–7 µL of sample and applied across the width of the gel (*see* **Notes 8–10**).
12. When all the samples are loaded, place a platinum electrode on each wick. Some commercial apparatus employ a small perspex plate along which the platinum electrodes are stretched and held taut. Good contact between the electrode and wick is maintained by applying a weight to the perspex plate.
13. Apply a potential difference of 500 V across the plate. This should give current of about 4–6 mA. After 10 min increase the current to 1000 V and then to 1500 V after

a further 10 min. A current of ~4–6 mA should be flowing in the gel at this stage, but this will slowly decrease with time as the gel focuses.

14. When the gel has been running for about 1 h, turn off the power and carefully remove the sample papers with a pair of tweezers. Most of the protein samples will have electrophoresed off the papers and be in the gel by now (*see* **Note 11**). Continue with a voltage of 1500 V for a further 1 h. During the period of electrophoresis, colored samples (myoglobin, cytochrome c, hemoglobin in any blood samples, and so on) will be seen to move through the gel and focus as sharp bands (*see* **Note 12**).
15. At the end of 2 h of electrophoresis, a current of about 0.5 mA will be detected (*see* **Note 13**). Remove the gel plate from the apparatus and *gently* wash it in fixing solution (200 mL) for 20 min (overvigorous washing can cause the gel to come away from the glass plate).

    Some precipitated protein bands should be observable in the gel at this stage (*see* **Note 14**). Pour off the fixing solution and wash the gel in destaining solution (100 mL) for 2 min and discard this solution (*see* **Note 15**). Add protein stain and gently agitate the gel for about 10 min. Pour off and discard the protein stain and wash the gel in destaining solution. Stained protein bands in the gel may be visualized on a light box and a permanent record may be obtained by simply leaving the gel to dry out on the glass plate overnight (*see* **Note 16**).
16. Should you wish to stain your gel for enzyme activity rather than staining for total protein, immediately following electrophoresis gently agitate the gel in an appropriate substrate solution (*see* **Note 7**, Chapter 20).

4. Notes

1. The sucrose is present to improve the mechanical stability of the gel. It also greatly reduces pH gradient drift.
2. The procedure described here uses photopolymerization to form the polyacrylamide gel. In the presence of light, riboflavin breaks down to give a free radical which initiates polymerization (Details of acrylamide polymerization are given in the introduction to Chapter 21). Ammonium persulphate /TEMED can be used to polymerize gels for IEF but there is always a danger that artefactual results can be produced by persulfate oxidation of proteins or ampholytes. Also, high levels of persulfate in the gel can cause distortion of protein bands (*see* **Note 10**).
3. This broad pH range is generally used because it allows one to look at the totality of proteins in a sample (but note that very basic proteins will run off the gel). However, ampholytes are available in a number of different pH ranges (e.g., 4–6, 5–7, 4–8, and so on) and can be used to expand the separation of proteins in a particular pH range. This is necessary when trying to resolve proteins with very similar pI values. Using the narrower ranges it is possible to separate proteins that differ in their pI values by as little as 0.01 of a pH unit.

4. Ensure that your light box does not generate much heat as this can dry out the gel quite easily by evaporation through any small gaps at the joints of the electrical insulation tape. If your light box is a warm one, stand it on its side and stand the gels adjacent to the box.
5. It is not at all obvious when a gel has set. However, if there are any small bubbles on the gel (these can occur particularly around the edges of the tape) polymerization can be observed by holding the gel up to the light and observing a "halo" around the bubble. This is caused by a region of unpolymerized acrylamide around the bubble that has been prevented from polymerizing by oxygen in the bubble. It is often convenient to introduce a small bubble at the end of the gel to help observe polymerization.
6. If the gel stays on the sheet of glass that does not contain the tape, discard the gel. Although usable for electrofocusing you will invariably find that the gel comes off the glass and rolls up into an unmanageable "scroll" during staining/destaining. To ensure that the gel adheres to the glass plate that has the tape on it, it is often useful to siliconize (e.g., with trimethyl silane) the upper glass plate before pouring the gel.
7. The strips must be fully wetted but must not leave a puddle of liquid when laid on the gel. (Note that in some apparatus designs application of the electrode applies pressure to the wicks, which can expel liquid.)
8. The filter paper must be fully wetted but should have no surplus liquid on the surface. When loaded on the gel this can lead to puddles of liquid on the gel surface which distorts electrophoresis in this region. The most appropriate volume depends on the absorbancy of the filter paper being used but about 5 µL is normally appropriate. For pure proteins load approx 0.5–1.0 µg of protein. The loading for complex mixtures will have to be done by trial and error.
9. Theoretically, samples can be loaded anywhere between the anode or cathode. However, if one knows approximately where the bands will focus it is best not to load the samples at this point since this can cause some distortion of the band. Similarly, protein stability is a consideration. For example, if a particular protein is easily denatured at acid pH values, then cathodal application would be appropriate.
10. The most common problem with IEF is distortions in the pH gradient. This produces wavy bands in the focused pattern. Causes are various, including poor electrical contact between electrode and electrode strips, variations in slab thickness, uneven wetting of electrode strips, and insufficient cooling leading to hot spots. However, the most common cause is excessive salt in the sample. If necessary, therefore, samples should be desalted by gel filtration or dialysis before running the gel.
11. Although not absolutely essential, removal of sample strips at this stage is encouraged since bands that focus in the region of these strips can be

distorted if strips are not removed. Take care not to make a hole in the gel when removing the strips. Use blunt tweezers (forceps) rather than pointed ones. When originally loading the samples it can be advantageous to leave one corner of the filter strip slightly raised from the surface to facilitate later removal with tweezers.

12. It is indeed good idea to include two or three blood samples in any run to act as markers and to confirm that electrophoresis is proceeding satisfactorily. Samples should be prepared by diluting a drop of blood approx 1:100 with distilled water to effect lysis of the erythrocytes. This solution should be pale cherry in color. During electrophoresis, the red hemoglobin will be seen to electrophorese off the filter paper into the gel and ultimately focus in the central region of the gel (pH 3.5–10 range). If samples are loaded from each end of the gel, when they have both focused in the sample place in the middle of the gel one can be fairly certain that isoelectrofocusing is occurring and indeed that the run is probably complete.
13. Theoretically, when the gel is fully focused, there should be no charged species to carry a current in the gel. In practice there is always a slow drift of buffer in the gel (electro-endomosis) resulting in a small (0.5 mA) current even when gels are fully focused. Blood samples (*see* **Note 9**) loaded as markers can provide additional confirmation that focusing is completed.
14. It is not possible to stain the IEF gel with protein stain immediately following electrophoresis since the ampholytes will stain giving a uniformly blue gel. The fixing step allows the separated proteins to be precipitated in the gel, while washing out the still soluble ampholytes.
15. This brief wash is important. If stain is added to the gel still wet with fixing solution, a certain amount of protein stain will precipitate out. This brief washing step prevents this.
16. If you wish to determine the isoelectric point of a protein in a sample, then the easiest way is to run a mixture of proteins of known pI in an adjacent track (such mixtures are commercially available). Some commercially available kits comprise totally colored compounds that also allows one to monitor the focusing as it occurs. However, it is just as easy to prepare ones own mixture from individual purified proteins. When stained, plot a graph of protein pI vs distance from an electrode to give a calibration graph. The distance moved by the unknown protein is also measured and its pI read from the graph. Alternatively, a blank track can be left adjacent to the sample. This is cut out prior to staining the gel and cut into 1 mm slices. Each slice is then homogenized in 1 mL of water and the pH of the resultant solution measured with a micro electrode. In this way a pH vs distance calibration graph is again produced.

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Serial Immobilized pH Gradient Isoelectric Focusing over pH 4–9

Slobodan Poznanović, Wojciech Wozny, Helmut Zengerling, Gerhard P. Schwall, and Michael A. Cahill

1. Introduction

It has long been apparent that the separation capacity of conventional 18 cm IPGs as the first separation step in two dimensional polyacrylamide gel electrophoresis (2D-PAGE) was theoretically incapable of separating the complexity that is to be expected by the protein species of a mammalian cell line or tissue ***(1)***. We first attempted to improve the resolution of 2D-PAGE by developing IPGs of 54 cm in length ***(2).*** While this strategy was successful, it required the labor-intensive preparation and batchwise validation of own-caste IPGs for each experiment. Subsequently we found that qualitatively similar excellent results could be obtained by placing commercially available IPGs in series (end to end) during IEF. We submitted this method as a patent application on September 12, 2003 (WO2005026715, but since allowed to lapse in all countries but Germany) and published it in the scientific literature in August 2005 ***(3).*** The main advantage of this analytical strategy is that less sample is consumed per high resolution protein separation as compared to the alternative use of conventional combinations of individual narrow pH range IPGs for IEF. Thus, the system is well suited to the optimal analysis of samples that are limited in amount, as we have since demonstrated in a number of analytical investigations, including cases where samples from small human clinical biopsies were employed ***(4–6).*** Farhoud and colleagues independently published an essentially identical serial IEF method and device ***(7),*** also emphasizing the maximal resolution and yield of minimally consumed scarce samples. That paper was submitted for publication in July 2005, four months after the publication of our patent application.

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2. Materials

2.1. Casting Polyacrylamide Bridges

1. Glass plates for casting polyacrylamide gels. 200 × 260 × 0.5 mm, U-frame and 200 × 260 × 4 mm glass plate (GE Healthcare 80-1106-87; 18-1102-47).
2. GelBond PAG film, 203 × 260 mm (GE Healthcare 80-1129-37).
3. PlusOne Acrylamide IEF (GE Healthcare 17-1300-01).
4. PlusOne N,N′-Methylene-bisacrylamide (GE Healthcare 17-1304-01).
5. PlusOne TEMED (GE Healthcare 17-1312-01).
6. PlusOne Ammonium Persulfate (GE Healthcare 17-1311-01).
7. PlusOne Repel-Silane ES (GE Healthcare 17-1332-01).
8. Kimtech Science Precision Wipes, Kimberly Clark Product Code: 75512.

2.2. Rehydration of IPG Strips

1. Immobiline DryStrip pH 4.0–5.0, 18 cm (GE Healthcare 17-6001-84).
2. Immobiline DryStrip pH 5.0–6.0, 18 cm (GE Healthcare 17-6001-86).
3. Immobiline DryStrip pH 6–9, 18 cm (GE Healthcare 17-6001-88).
4. IEF buffer: 7 M urea, 2 M thiourea, 1% Triton X-100, 10% glycerol, 4% CHAPS, 1% DTT.
5. IPG Buffer pH 3.5–5.0 (GE Healthcare 17-6002-02).
6. IPG Buffer pH 5.0–6.0 (GE Healthcare 17-6002-05).
7. Servalyte 6–9 (Serva Heidelberg 42913.01).
8. Immobiline DryStrip Reswelling Tray, for 7–18 cm IPG strips (GE Healthcare 80-6371-84).
9. Flat laboratory forceps suitable for IPG strip manipulation.
10. Sharp laboratory scissors (Stainless steel).

2.3. Assembly of Daisy Chain IPGs

1. Immobiline DryStrip Cover Fluid (GE Healthcare 17-1335-01).
2. Acrylamide bridges (*see* **Note 1**).

2.4. Isoelectric Focussing

1. Self-made IEF device (*see* **Note 2**).

3. Methods

In this methodical report only the serial IEF methodology is thoroughly described. We assume that the experimenter is already able to perform conventional 2D-PAGE using IPGs. Introductory material and references for this area can be found in the series of articles by Görg and colleagues (***8–10***); *see also* Chapter 35. The individual IPGs are best subsequently electrophoresed by SDS-PAGE and stained according to the protocols already established/optimized in your own laboratory for conventional single IPG-IEF.

3.1. Casting Polyacrylamide Bridges

1. Prior to use, wash the glass plates thoroughly with diluted detergent, followed by acetone, and then rinse with deionized water and air dry (*see* **Note 3**).
2. Siliconize glass plate bearing U-frame (*see* **Note 4**).
3. Wash Gel Bond PAG films 3 × 10 min with deionized water prior to use (*see* **Note 5**).
4. Assemble gel casting cassette prior to use and leave it in vertical position (*see* **Note 6**).
5. Prepare 4% acrylamide solution according to the given recipe (*see* **Note 7**). Fill the assembled casting cassette with approximately 23 mL (or less) of acrylamide solution and polymerize for 15 min at room temperature (*see* **Note 8**). Subsequently polymerize the gel for 1 hour at 50°C in a heating cabinet (*see* **Note 9**).
6. Cool down the gel (*see* **Note 10**) and cut them into 4 mm wide strips (*see* **Note 11**).
7. Store Acrylamide bridges at −20°C until use (*see* **Note 12**).

3.2. Rehydration of IPG Strips

1. Prepare IEF rehydration buffer or thaw from −20°C previously prepared buffer.
2. Rehydrate IPG strips pH 4–5, 5–6 and 6–9 according to standard protocol as you would for individual IPGs of these pH ranges (*see* **Note 13**).
3. Rehydrate bridges in IEF buffer with IPG buffer mixture (*see* **Note 14**).

3.3. Assembly of Daisy Chain IPGs

1. Assemble the IEF chamber as you routinely do with Multiphor IEF chamber **(Fig. 1).**
2. Fill the IEF chamber with Immobiline DryStrip Cover Fluid or Paraffin oil you use for standard IEF of IPG strips (*see* **Note 15**).
3. Assemble IPG strips from acidic to basic (from left to right) (*see* **Note 16**).
4. Cut bridges in 1 – 1.5 cm long pieces.
5. Gently apply the bridging gels to span between the IPG strips, briefly applying slight pressure to facilitate electrical contact between the aqueous zones **(Fig. 2).**

3.4. Isoelectric Focusing

1. Voltages employed for IEF are parameterized in **Table 1.**
2. After IEF the IPGs are handled identically to IPGs in conventional single gel 2D-PAGE.

4. Notes

1. Casting of “bridges” should be done at least one day before one plans to start daisy chain. The best is to cast bridges in advance, few days before, in sufficient amount and to store them at −20°C until use.
2. Our description of the self-made IEF device has been published (*3*). Farhoud and colleagues independently published an essentially identical serial IEF method and device (*7*). We recommend consulting both of these cited references before attempting to construct an own high voltage IEF device, and certainly not to even consider this without the assistance of suitably qualified

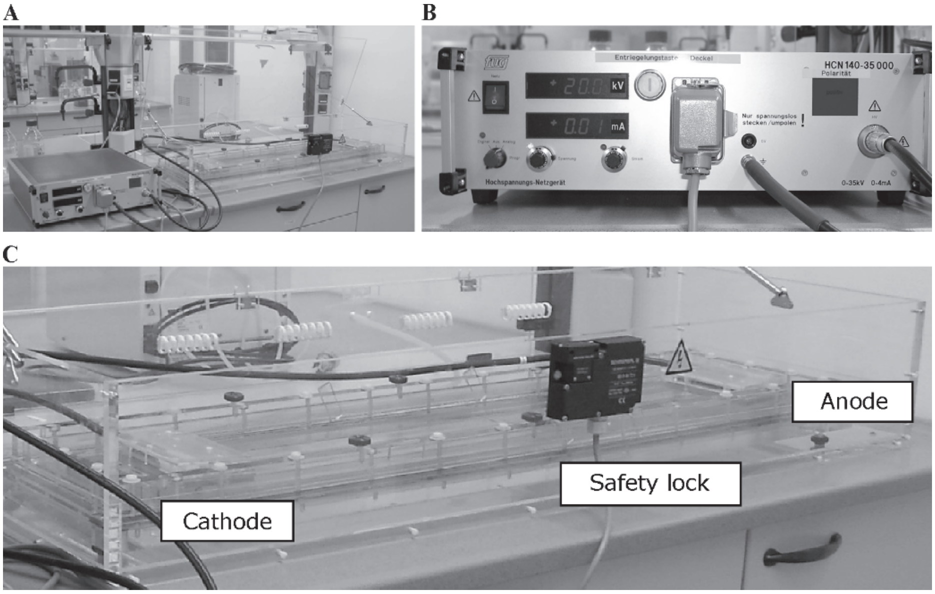

Fig.1. Working prototype long IEF chamber (for IEF up to 96 cm). **(A)** The IEF chamber and power supply. The safety lid is open and the temperature regulating water cooler is behind the device. **(B)** The power supply, running at 20 kV (for more rapid IEF, alternatively to Table 1)**.** **(C)** Detail view of the IEF chamber. The safety lock is connected via a time-delayed circuit to an unlocking mechanism on the power supply, and can be opened only 5 minutes after the power has been switched off. Other features include**:** variable IEF length: 18 – 100 cm, integrated cooling surface, silicon oil coolant is an electrical insulator, 35 kV power supply, 0.05 mA adjustable steps, high voltage cables for 35 kV, custom designed electrodes for 35 kV, and electrically insulating safety chamber surrounds IEF chamber.

electrical and/or engineering skill. All electrical circuitry in the self-made IEF chamber was configured according to German regulation VDE 0411 concerning the use of voltages over 10,000 V. The IEF chamber was based upon a water-cooled aluminum surface of about 1100 mm × 240 mm. A shallow glass tray was placed onto the metal surface, and cooled by application of mineral oil between metal and glass to permit heat transfer. The glass tray itself is 940 mm × 145 mm, and has walls that are 18 mm high, resembling a long Pharmacia Multiphore DryStrip tray. The IPGs are placed into this tray as described below and electrodes are placed at each end. These electrodes have cables rated >10,000 V and lead to a HCN 140–35000 power supply which is programmable at 0–4 mA in 50 µA steps (F.u.G. Elektronic, Rosenheim Germany). The cooled aluminum surface with glass tray and electrodes were completely contained in a large Plexiglas (acrylic glass/polymethyl methacrylate) case with a hinging top panel that serves as a locking lid. Plexiglas was

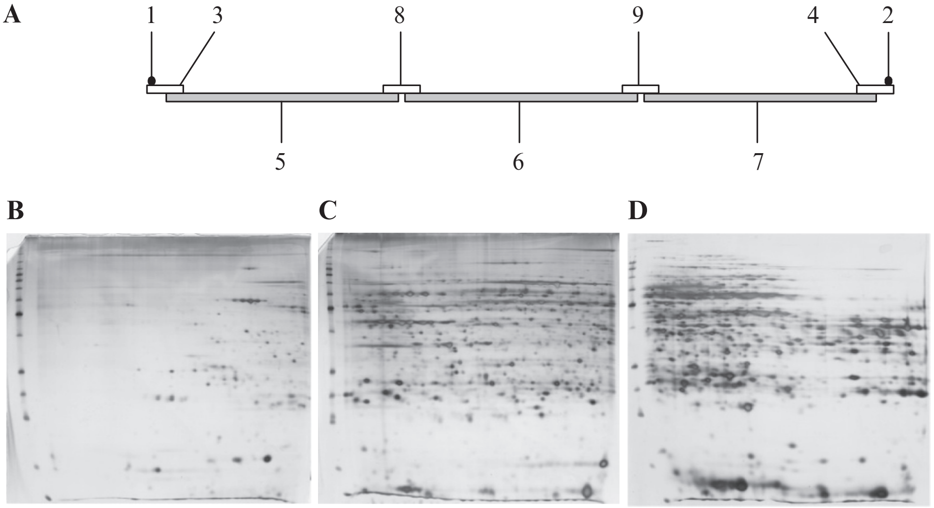

Fig. 2. **(A)** Schematic "Daisy Chain" IPG serial IEF design. *1,* Anode; *2,* Cathode; *3 and 4,* Water-soaked paper wick; *5,* IPG pH 4.0 – 5.0; *6,* IPG pH 5.0 – 6.0; *7,* IPG pH 6.0 – 9.0; *8,* Bridge between 5 and 6; *9,* Bridge between 6 and 7. **(B-D)** Silver stained 2D-PAGE gels of 250 µg of swine liver proteins from commercial IPGs (Amersham) electrophoresed according to A in 12% SDS-PAGE gels. Molecular weight markers: kDa 220; 160; 120; 100; 90; 80; 70; 60; 50 (fat); 40; 30; 25; 20 (fat/double); 15.

chosen for its excellent properties of electrical insulation. The hinged lid was additionally fitted with an opening safety mechanism whose catch was electronically linked to the power supply. This had been internally custom modified during self-construction to prevent the lid from opening until voltage levels had dropped to safe levels. The joints of the Plexiglas case were also waterproofed with silicon and the case was of sufficient volume that any leakage from the water-cooling system would be retained inside the Plexiglas vessel, and would not come into contact with the electrodes. These aspects are mentioned to emphasize that the personal safety of the experimenter is dependent

Table 1.
Serial IEF Regime

150 V	30 min
300 V	30 min
600 V	60 min
3000 V	up to 10000 Vh
10000 V	up to 120000 Vh
1000 V	for 1440 min (stand by)

upon the technical design of the IEF device. Because commercially manufactured devices are not available, local governmental safety regulations (and common sense) should be strictly adhered to as the minimum standard in the manufacture of a suitable apparatus.

3. All glass plates must be absolutely clean and free of fat and detergents.
4. Treatment of plates with Repel-Silane enables an easier removal of glass plate from cast gels. Repel-Silane ES is a 2% solution of dimethyldichlorosilane dissolved in octamethyl cyclo-octasilane, a low toxicity, and environmentally safe solvent. Pipette 1–2 ml of repel silane on the glass plate with the U-frame and distribute it evenly over whole surface with a lint-free paper (e.g., Kimwipe). Let it dry for a few minutes, rinse with ethanol and repeat procedure once again. This procedure must be repeated occasionally in order to prevent the gels from sticking to the glass plates. Repel silane glass plates can be re-used, but never mix them with normal glass plates! When not in use siliconized glass plates should be stored separately from non-siliconized ones. Pack the plates face to face with a piece of tissue paper between them to avoid contamination or scratching of the glass. To facilitate efficient siliconization of the surface and thereby avoid sticking of gels to the glass plates, it is recommended that the procedure should be repeated at least twice in succession.
5. Always wash with the hydrophilic side upwards. Gel Bond PAG film is packed with the treated side up, with paper interleaving to protect the treated surface. If uncertain, the hydrophilic side can be confirmed by applying a drop of water and noting that it spreads on the hydrophilic side, but beads up on the hydrophobic side. Always handle Gel Bond PAG film by the edges and with gloves to avoid getting fingerprints on the treated surface. Note that PAG film is both light and heat sensitive. It should be stored under cool dark conditions, and you should be aware that after prolonged or inappropriate storage the acrylamide groups may be degraded, and your gels will not be able to polymerize to the surface satisfactorily.
6. Our casting cassette consists of two glass plates of 200 × 260 mm. One of these is covered with the Gel Bond PAG film and the other contains a 0.5 mm U-frame. The frame is made of silicon rubber enclosing 0.5 mm metal balls. The cassette is assembled as originally described by Görg and colleagues (http://www.weihenstephan.de/blm/deg/2D-Manual_06.pdf) (*8–10*). The cleaned glass plate without the U-frame is moistened with a few drops of water, and the Gel Bond PAG film, hydrophilic side upwards, is placed onto the wet glass surface. The surface of the Gel Bond PAG film is covered by a piece of Kimtech tissue paper and excess water is expelled with a roller as originally described by Görg and colleagues. Place the glass plate with the U-frame (0.5 mm thick) on top of the Gel Bond PAG film and clamp the cassette together.

7. Chemicals used for preparation of gel should be of the highest possible quality. We use IEF grade acrylamide purchased in powder form. A stock solution (30% T; 2,7% C) is always freshly prepared or occasionally stored at +4°C for a maximum of one week. We have found it unnecessary to use ion exchange resin for preparation of such stock solutions. Omission of ion exchange avoids potential contamination of the acrylamide solution with impurities from the resin preparations. Stock solution (30% T; 2,7% C): Dissolve 29,18 g of Acrylamide and 0,81 g of bisacrylamide in MilliQ water and fill up to 100 mL. Solution should be filtered and stored at +4 °C. To make the 4% gels add 4 ml acrylamide stock to 26 mL H_2O, then add 30 µL of freshly prepared 40% ammonium persulfate and 20 µL TEMED.
8. The gel can be caste using several different methods. The most important point is to fill slowly to avoid the formation of bubbles. We apply the acrylamide mixture slowly and steadily to one side of the casting chamber with a 10 mL pipette, initially tilting the cassette about 40° to run the gel down that side. This should obviously not be performed too slowly because of the advancing polymerization reaction itself.
9. Although the acrylamide mixture should have gelled by this stage, during polymerization in the oven the caste gel in its cassette should remain in the vertical position, this way avoiding any possible movement and leakage of the gel.
10. After polymerization at 50°C allow the gel to cool down to RT for at least 15 min. Disassemble the cassette by inserting a spatula between gel and glass plate and gently but firmly levering the plates apart. Remove the support film with attached gel carefully and wash the PAG film and gel 4 × 15 min with 500 ml MilliQ water to remove unpolymerized acrylamide monomers. Finally incubate the gel in 2% Glycerol for 30 min. Dry the gel overnight in a hood or with a fan or hair-dryer for a few hours. We prefer drying overnight, avoiding potential dust (and keratin) contamination which may occur by drying with a forced air flow, such as caused by a hair dryer or electric fan.
11. The gel can be cut into strips using an office paper cutter, as suggested by Görg. Cleaner edges can be achieved by scission with an industrial paper cutting machine.
12. The bridge gels, consisting only of polyacrylamide without buffers at this stage, are quite stable. We have used precast bridging gels stored sealed at −20°C for more than one year, after which the supply was exhausted.
13. Rehydration of strips should be done according to the instruction for the individual strips. There can be different methods of sample application for daisy chain experiments, e.g.:
    - sample application by in-gel rehydration.
    - sample application via wick- or cup-loading
    - sample application via bridge loading

Table 2
Buffers for IPG Rehydration

pH of IPG strip	Case A (in-gel rehydration)	Case B (wick application)
4–5	Sample + IEF buffer with 0.8% of IPG buffer pH 3.5–5.0	IEF buffer with 0.8 % of IPG buffer pH 3.5–5.0
5–6	IEF buffer with 0.8 % of IPG buffer pH 5.0–6.0	IEF buffer with 0.8 % of IPG buffer pH 5.0–6.0
6–9	IEF buffer with 0.8 % of Servalyte CA pH 6.0–9.0	IEF buffer with 0.8 % of Servalyte CA pH 6.0–9.0
Bridges	IEF buffer with 0.8% mix of IPG buffers 3.5–5.0; 5.0–6.0 and Servalyte 6.0–9.0, **1:1:1**	IEF buffer with 0.8% mix of IPG buffers 3.5–5.0; 5.0–6.0 and Servalyte 6.0–9.0, **1:1:1**

The best result depend upon the nature of the proteins in your sample. In the first case the sample is loaded by the rehydration method into the IPG strip pH 4–5 or 5–6 depending on the sample. The other IPG strips for the daisy chain are rehydrated with IEF buffer containing the appropriate corresponding IPG buffer.

The optimum method of loading the strips by rehydration will also be sample-specific, with better 2D-PAGE samples obtained by rehydration loading into the basic IPG, the acidic IPG, or all IPGs for different samples.

When we apply sample via the wick then all single gels are rehydrated in their appropriate IEF buffer with corresponding individual pH-specific IPG buffer. In all cases for each IPG strip pH gradient we used 0.8% of corresponding IPG buffer.

For specific samples better results can be obtained by bridge loading. The optimum position of the loading bridge is sample dependent and should be empirically determined.

A tabular presentation of appropriate conditions is depicted in Table 2.

14. Bridges were always rehydrated in IEF buffer containing 0.8% of mixture all used IPG buffers and Servalyte in relation 1:1:1. In all cases rehydration was done on the lab bench overnight at room temperature. Shorter rehydration steps can be employed, although we advise rehydration for more than 5 hours.
15. The IPG holding tray chamber should be filled with oil sufficiently that the IPG strips and paper wicks are submerged. It may be more convenient to first fill the IPG tray with oil and then lay the strips gently down into the oil.
16. To simplify assembling of a daisy chain in the IEF tray we use laminated grid paper positioned under the tray. Try to position the IPGs as closely as possible end-to-end, avoiding any overlap. To position the IPGs we suggest cutting the plastic ends of the IPG strips before alignment, according to the following schema:

- IPG strip pH 4–5: Cut the plastic end close to the gel at the cathodic ("–" or basic) end.
- IPG strip pH 5–6: Cut the plastic ends close to the gel at both the anodic ("+" or acidic) and cathodic ("–" or basic) ends.
- IPG strip pH 6–9: Cut the plastic end close to the gel at the anodic ("+" or acidic) end.

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Radiolabeling of Eukaryotic Cells and Subsequent Preparation for 2-Dimensional Electrophoresis

Nick Bizios

1. Introduction

Two dimensional polyacrylamide gel electrophoresis (2-DE) provides not only the ability to resolve and quantify thousands of proteins, but it also gives those in research and industry the ability to monitor in-process protein purification, quickly and easily ***(1)***. It can become a valuable tool in process analytical technology (PAT) in which the goal is to understand and control a process in order to build in quality. 2-DE is also used to identify variability in protein expression in a variety of cell lines ***(2,3)***.

It is known that many parameters and laboratory conditions can influence the resolution of proteins on 2-DE, such as pH range of carrier ampholytes used, the quality of reagents and equipment used, temperature, voltage, and the skill of the researcher or technician. Even though there are a multitude of vendors that supply premade reagents, there is pride bestowed to the technician or researcher performing their work.

One of the biggest obstacles one may encounter prior to performing 2-DE is the proper documentation and validation of a Standard Operating Procedure, or SOP. This falls under the category of current Good Laboratory Practices (cGLPs) and or current Good Manufacturing Practices (cGMPs). Without the proper documentation of reagents used, such as Certificates of Analysis (readily supplied by the supplier's Quality Control/Assurance department), adherence to expiration dates of commercially prepared reagents, implementation of expiration dates of reagents prepared in the laboratory and the adherence of SOPs (*see* **Note 1**) the ability of generating repeatable and consistent results falls dramatically. However, the radiolabeling and preparation or eukaryotic cells for 2-D PAGE may be considered paramount in obtaining consistent results. Without proper

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radiolabeling and preparation, how would one find subsequent spots of interest and perform quantitative analysis?

This chapter will describe a general and robust method for the labeling methionine-containing proteins, phosphorylation labeling, and subsequent lysate preparation for 2-DE that have been modified from Garrels ***(4)*** and Garrels and Franza ***(5)***. The Jurkat T-lymphoblast cell line is used as an example.

A larger number of protocols have been published for the solubilization and sample preparation of eukaryotic cell lines and tissues for 2-DE. One of the best places for additional protocols is found at the Geneva University Hospital's Electrophoresis laboratory, which can be accessed at http;//expasy.hcuge.ch/ch2d/technical-info.html.

2. Materials

2.1. Equipment

1. 0.2µm filters (*see* **Note 2**)
2. Heat block or 100°C water bath

2.2. Reagents

1. Complete culture medium: 90% RPMI-1640, 10% fetal bovine serum (FBS), streptomycin-penicillin: Mix 990mL of RPMI-1640 with 100mL of FBS, and add 10mL of streptomycin-penicillin (100X). Cold-filter-sterilize using a 0.2µm filter. Store at 4°C. Maintain the Jurkat T-lymphoblast at a concentration of 10^5–10^6 cells/mL (*see* **Note 3**).
2. Methionine-free medium: 90% methionine-free RPMI-1640, 10% dialyzed FBS (dFBS): Mix 990mL of methionine-free RPMI-1640 with 100mL of dFBS. Cold-filter-sterilize using 0.2µm filter. Store at 4°C.
3. Sodium phosphate-free medium: 90% sodium phosphate-free RPMI-1640, 10% FBS: Mix 90% sodium phosphate free RPMI with 10% FBS. Cold-filter-sterilize. Store at 4°C.
4. Redivue L-[^{35}S]methionine (cell labeling grade) (GE Healthcare)
5. ^{32}P-orthophospahte (GE Healthcare).
6. Phosphate buffered saline (PBS): Mix 8g NaCl, 0.2KCl, 1.44g Na_2HPO_4, and 0.24 grams KH_2PO_4 in 800mL of dH_2O) and adjust to 7.4 with HCl. Add dH_2O to a final volume of 1000mL and autoclave. Store at room temperature.
7. Dilute SDS (dSDS): 0.3% SDS, 1% β-mercaptoethanol (β-ME), 0.05M Tris-HCl, pH 8.0. In a cold room, mix 3.0g of SDS, 4.44g of Tris-HCl, 2.65g Tris base, 10mL of β-ME in dH_2O, and adjust the final to 1L with dH_2O. Aliquot 500mL into microcentrifuge tubes and store at -70°C.
8. DNase/RNase solution: 1mg/mL DNase I, 0.5mg/mL RNase A, 0.5*M* Tris, 0.05*M* $MgCl_2$, pH 7.0. Thaw RNase, Tris, and $MgCl_2$ stocks, and thoroughly mix 2.5mg of RNase A (Worthington Enzymes), 1585µL of 1.5*M* Tris-HCl, 80µL of 1.5 Tris base, 250µL of 1.0*M* $MgCl_2$, and 2960µL of dH_2O. Mix the liquids, and add 5mg

DNase I (Worthington Biochemical). Do not filter. Keep cool while dispensing into microcentrifuge tubes. Makes 50 µL aliquots, and store at −70°C.

9. 1.5 *M* Tris-HCl solution: Weigh out 11.8 g of desiccated Tris-HCl, and add 41.4 g dH_2O. Mix well and filter through 0.2 µm filter. Aliquot into microcentrifuge tubes, and store at −70°C.
10. 1.5 *M* Tris base solution: Weigh out 9.09 g of Tris base, and add 41.4 g of dH_2O. Mix and filter through 0.2 µm filter. Aliquot into microcentrifuge tubes, and store at −70°C.
11. 1.0 *M* $MgCl_2$ solution: Weigh out 30.3 g of $MgCl_2$, and add 85.9 g of dH_2O. Mix and filter through 0.2 µm filter. Aliquot into microcentrifuge tubes, and store at −70°C.
12. Sample buffer solution (SB): 9.95 M urea, 4.0% Igepal CA-630 (Sigma), 2% 6.0–8.0 ampholytes, 100 m*M* dithiothreitol (DTT). Mix 59.7 g urea, 44.9 g dH_2O, 4.0 g Igepal CA-630, 5.5 g of pH 5.0 to 8.0 ampholytes (GE Healthcare), 1.54 g of DTT (Calbiochem) in this order in a 30° to 37° water bath just long enough to dissolve the urea. Filter through a 0.2 µm filter, and aliquot 1 mL into microcentrifuge tubes. Snap-freeze in liquid nitrogen, and store at −70°C.
13. Sample buffer with SDS solution (SBS): 9.95 M urea, 4.0% Igepal CA-630 (Sigma), 0.3 % SDS, 2% 6.0–8.0 ampholytes, 100 m*M* dithiothreitol (DTT). Mix 59.7 g urea, 44.9 g dH_2O, 4.0 g Igepal CA-630, 0.3 g SDS, 5.5 g of pH 5.0 to 8.0 ampholytes, 1.54 g of DTT in this order in a 30° to 37° water bath just long enough to dissolve the urea. Filter through a 0.2 µm filter, and aliquot 1 mL into microcentrifuge tubes. Snap-freeze in liquid nitrogen, and store at −70°C.

3. Methods

3.1. ^{35}S-Labeling (see Note 4)

1. Jurkat T-lymphocytes are labeled for 3–24 h in methionine-free media containing 50–250 µCi/mL of ^{35}S.
2. Follow cell lysate protocol (**Subheading 3.3.,**).

3.2. ^{32}P-Labeling (see Note 3)

1. Add 100 µCi/mL of ^{32}P for up to 3 h to cells that are in phosphate-free medium (*see* **Note 5**).

3.3. Whole-Cell Lysate Preparation

1. Wash cells with PBS three times in a microcentrifuge tube.
2. Add an equal volume of hot (100°C) dSDS solution to the pellet.
3. Boil tube (100°C) for 1–3 min.
4. Cool in an ice bath (*see* **Note 6**).
5. Add one-tenth (1/10) volume of DNase/RNase solution.
6. Gently vortex for several minutes to avoid foaming. The sample should lose its viscosity, and the solution should look clear. If not, then add more dSDS and DNase/RNase solution (*see* **Note 7**).
7. Snap-freeze in liquid nitrogen, and store at −70°C. Samples may be kept for up to 6 mo at −70°C.

3.4. Preparing the Sample for 2D-PAGE: Vacuum Drying

1. Lyophilize sample (frozen at −70°C) in a Speed Vac using no or low heat until dry *see* **Note 8**).
2. Add SB solution to the sample equal to that of the original dSDS sample volume, and mix thoroughly.
3. Heat sample to 37°C for a short period if necessary (*see* **Note 9**).
4. Store at −70°C. Samples can be kept for up to 6 mo at −70°C.
5. Radioisotope incorporation in the sample may now be determined by TCA precipitation.
6. Recommended first-dimension load is 500,000 dpm for ^{35}S-labeled proteins and 200,000 dpm for ^{32}P-labeled proteins.
7. If necessary, the sample is diluted and mixed thoroughly with SBS solution before loading onto the first-dimension gel (*see* **Note 9**).

4. Notes

1. cGLPs and cGMPs will help with all aspects in a research facility. Industry will have Quality Assurance/Control departments to oversee the implementation of these practices. Smaller research labs may wish to assign or hire a person to oversee these practices and adhere to.
2. Small syringe type 0.2 µm filters are best acquired from Nalgene while larger scale filters requiring a pump are best supplied from Sartorius-Stedim Biotech, Pall or Millipore.
3. Maintain Jurkat T-lymphocytes in complete culture medium supplemented with streptomycin/penicillin, in a humidified incubator with 95% air and 5% CO_2 at 37°C at a concentration of 10^5-10^6 cells/mL.
4. It is extremely important that all radioactive work be performed with the utmost care, and according to your institutional and local guidelines.
5. It may be necessary to preincubate the cells. Preincubating the cells at a density of 1–10 × 10^6 cells/mL for 30 min in sodium-free, phosphate-free medium works well.
6. Sample preparation should be done quickly once to avoid degradation by proteases.
7. Keep salt concentrations as low as possible, high concentrations (>150 m*M*) of NaCl, KCl, and other salts cause streaking problems, as do lower concentrations of phosphate and charged buffers. Dialyzing samples to remove salts and other low-mol-wt substances is recommended.
8. Larger sample volumes may be prepared using a large scale commercial lyophilizer in order to prepare "internal standards" for each run.
9. After dissolving the sample in SB solution or diluting in SBS solution, it is imperative that the sample is not subjected to temperatures above 37°C. At extreme temperature (>40°C), the urea in SB and SBS will

cause carbamylation. Charged isoforms will be generated by isocyanates formed by the decomposition of urea.

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Two-Dimensional Polyacrylamide Gel Electrophoresis of Proteins Using Carrier Ampholyte pH Gradients in the First Dimension

Patricia Gravel

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is the only method currently available for the simultaneous separation of thousands of protein. This method separates individual proteins and polypeptide chains according to their isoelectric point and molecular weight. The 2-D PAGE technology can be used for several applications, from which; separation of complex protein mixture into their individual polypeptide components; comparison of protein expression profiles of sample pairs (normal versus transformed cells, cells at different stages of growth or differentiation, etc.).

This chapter describes the protocol for 2-D PAGE using carrier ampholyte pH gradient gel for the isoelectric focusing separation (pH gradient 3.5–10) and a polyacrylamide gradient gel for the second dimension ***(1,2)***.

For many years the 2-D PAGE technology relied on the use of carrier ampholytes to establish the pH gradient. This traditional technique has proven to be difficult in the hands of many because of the lack of reproducibility created by uncontrolable variations in the batches of ampholytes used to generate the pH gradients. This problem was solved by using immobilised pH gradients (IPG) (*see* Chapter 35), where the compounds used to set up the pH gradient are chemically immobilized and so the gradient is stable. Another advantage is that a larger amount of protein could be used in the separation for micropreparative runs. The availability of commercial precast IPG gels in a variety of narrow and broad pH ranges, as well as precast SDS-PAGE gels has lead also to major advances in protein separation, display and protein characterization ***(3).*** 2-D PAGE reference maps are now available over the World-Wide Web ***(4, 5)***.

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2. Materials

2.1. Preparation of Samples

1. Lysis solution A:10 % (w/v) SDS (sodium dodecyl sulfate) and 2.32 % (w/v) DTE (1,4- dithio-erythritol) in distilled water (dH_2O).
2. Lysis solution B:5.4 g urea (9.0 *M*) (must be solubilized in water at warm temperature, around 35°C), 0.1 g DTE (65 m*M*), 0.4 g CHAPS (cholamidopropyldimethylhydroxypropane sulfonate) (65 m*M*), 0.5 ml of Ampholines pH range 3.5–10 (5% v/v) made to 10 ml with dH_2O.

These solutions can be aliquoted and stored at –20°C for many months.

2.2. Isoelectric Focusing (IEF)

1. IEF is performed with the Tube Cell Model 175 (Bio-Rad) (see **Fig. 1**) and with glass capillary tubes (1.0 – 1.4 mm internal diameter and 210 mm long).

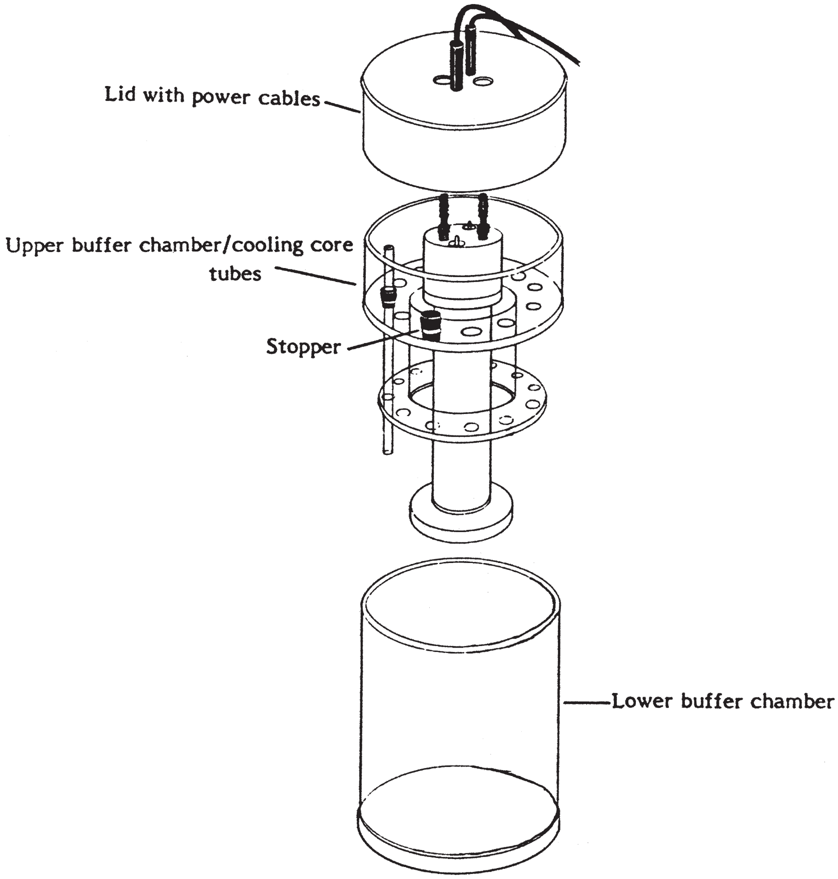
Lid with power cables
Upper buffer chamber/cooling core tubes
Stopper
Lower buffer chamber

Fig. 1. Tube Cell Model 175 (Bio-Rad) for the isoelectric focusing with carrier ampholyte pH gradient capillary gels.

Ampholytes pH 4–8 and Ampholytes pH 3.5–10 are from BDH (Poole, England).

2. Stock solution of acrylamide: 30% (w/v) acrylamide, 0.8% (w/v) PDA (piperazine diacrylamide) in dH_2O. This solution should be stored in the dark at 4°C for 1 to 2 months.
3. Cathodic buffer: 20 m*M* NaOH in dH_2O. This solution should be made fresh.
4. Anodic buffer: 6 m*M* H_3PO_4 in dH_2O. This solution should be made fresh.
5. Ammonium persulfate (APS) stock solution: 10% (w/v) APS in dH_2O. This solution should be stored at 4°C, protected from light and made fresh every 2 to 3 weeks.
6. SDS stock solution: 10% (w/v) SDS in dH_2O. This solution can be stored at room temperature.
7. Bromophenol blue stock solution: 0.05% (w/v) bromophenol blue in dH_2O. This solution can be stored at room temperature.
8. Capillary gel equilibration buffer:
   20 ml 0.5 *M* Tris-HCl pH 6.8
   40 ml 10% (w/v) SDS stock solution
   8 ml 0.05% (w/v) bromophenol blue stock solution
   72 ml dH_2O

This solution can be stored at room temperature for 2 to 3 months.

2.3. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. The Protean II chamber (Bio-Rad) is employed by us for SDS-PAGE. The gels (160 × 200 × 1.5 mm) are cast in the Protean II casting chamber (Bio-Rad). The gradient former is model 395 (Bio-Rad).
2. Running buffer: 50 m*M* Tris base, 384 m*M* glycine, 0.1% (w/v) SDS in dH_2O For 1 L: 6 g Tris base, 28.8 g glycine and 1 g SDS. Do not adjust pH. Fresh solution is made up for the upper tank. The lower tank running buffer can be retained for more than 6 months with the addition of 0.02% (w/v) sodium azide.
3. Sodium thiosulfate stock solution: 5% (w/v) sodium thiosulfate anhydrous in dH_2O. This solution should be stored at 4°C. (*See* **Note 1**.)
4. Silver staining: Proteins in the 2-D gel are stained with silver. We used the ammoniacal silver nitrate method modified by Hochstrasser et al. ***(6)*** and Rabilloud ***(7)***.

3. Method

3.1. Preparation of Protein Samples

Pellets of cells or tissue should be resuspended in 100 µl of 10 m*M* Tris-HCl pH 7.4 and sonicated on ice for 30 s. Add one volume of lysis solution B and mix. The mixed solution should not be warmed up above room temperature. Samples can be loaded directly onto the gels or stored at −80°C until needed. (*See* **Note 2** for plasma sample preparation and **Note 3** for optimal sample loading).

3.2. Isoelectric Focusing (IEF)

1. Draw a line at 16 cm on clean and dry glass capillary tubes (capillary should be cleaned with sulfochromic acid to eliminate all deposits). The remaining 0.5 cm of the capillary tubes is used to load the sample. Place each tube in a small glass test tube (tube of 5 ml) which will be filled with the isoelectric focusing gel solution. Connect the tops of each glass capillary tube to flexible plastic tubes joined together with a 1 ml plastic syringe.
2. At least twelve capillary gels can be cast with 11.5 ml of isoelectric focusing gel solution (800 µL of solution is needed per capillary).
   a. Prepare one milliliter of CHAPS 30% (w/v) and NP-40 (nonidet P-40) 10% (w/v) (0.3 g CHAPS and 0.1 g NP-40) and degase for 5 min.
   b. Separately, prepare a second solution: 10 g urea (7 ml of water is added to dissolve the urea at warm temperature, around 35°C), 2.5 ml of acrylamide stock solution, 0.6 ml of ampholytes pH 4–8, 0.4 ml of ampholytes pH 3.5–10 and 20 µL of TEMED.
   c. Mix the first solution (CHAPS, NP-40) with the second one. Degase the mixture and add 40 µL of APS 10% (w/v) stock solution. Pipette one ml of this isoelectric focusing gel solution into the glass test tubes (along the side walls in order to prevent the formation of air bubbles in the solution). Fill up the capillary tubes by slowly pulling the syringe (up to the height of 16 cm).
   d. After 2 h of polymerization at room temperature, pull the capillary tubes out of the glass test tubes. Clean and gently rub the bottom of each capillary gel with parafilm.
3. Fill the lower chamber with the anodic solution. Wet the external faces of the capillary tubes with water and insert them in the isoelectric focusing chamber. Load the samples on the top of the capillary (cathodic side). Generally, thirty to 40 µL of the final diluted sample is loaded using a 25 µL Hamilton syringe (*see* **Notes 3** and **4**).
4. Lay down the cathodic buffer solution at the top of the sample in the capillary tube and then fill the upper chamber. Connect the upper chamber to the cathode, and the lower chamber to the anode.
5. Electrical conditions for IEF are 200 V for 2 h, 500 V for 5 h and 1000 V overnight (16 h) at room temperature (*see* **Note 5**).
6. After IEF, remove the capillary tubes from the tank and force the gels out from the glass tube with a 1 ml syringe which is connected to a pipet tip and filled with water (*see* **Note 6**). Put the extruded capillary gels on the higher glass plate of the polyacrylamide gel (*see* **Subheading 3.3**). Residual water around the capillary gel should be soaked up with a filter paper. Put 140 µL of IEF equilibration buffer down on the capillary gel. Immediatly push the capillary gels between the glass plates of the polyacrylamide gel using a small spatula. Place the cathodic side (basic end) of the capillary gel at the right side of the polyacrylamide gel. Care should be taken to avoid the entrapment of air bubbles between the capillary and the polyacrylamide gels. It is not necessary to seal the capillary gels with agarose solution but the contact between the capillary gel and the 9–16% polyacrylamide gradient gel should be very tight.

3.3. SDS Polyacrylamide Gel Electrophoresis

1. In order to separate the majority of proteins, a 9–16% (w/v) polyacrylamide gradient gel is used for the second dimension. The gel is made as follows with 60 ml solution for a 9–16% (w/v) gel: 30 ml of 9% (w/v) acrylamide solution and 30 ml of 16% (w/v) acrylamide solution. Sixty milliliters are necessary to cast one gel of 1.5 mm × 200 mm × 160 mm (*see* **Note 7**).

Light solution:

9 ml of acrylamide stock solution (30% v/v)
7.5 ml 1.5 *M* Tris-HCl pH 8.8 (25% v/v)
150 µL 5% sodium thiosulfate solution (0.5% v/v)
15 µL TEMED (0.05% v/v)
13.2 ml water. This solution is degased and then 150 µL of 10% APS solution (0.5% v/v) is added.

Heavy solution:

16 ml of acrylamide stock solution (53% v/v)
7.5 ml 1.5 *M* Tris-HCl pH 8.8 (25% v/v)
150 µL of 5% sodium thiosulfate solution (0.5% v/v)
15 µL TEMED (0.05% v/v)
6.2 ml water. This solution is degased and then 150 µL of 10% APS solution (0.5% v/v) is added.

The gradient gel is formed using the Model 395 gradient Former (Bio-Rad) and a peristaltic pump. Immediately after the casting, the gels are gently over layered with a water-saturated 2-butanol solution using a 1 ml syringe. This procedure avoids the contact of the gel with air and allows to obtain a regular surface of the gel. Caution should be taken to avoid mixing the 2-butanol with the gel solution. The gels are stored overnight at room temperature to assure complete polymerization.

2. Prior to the second dimension separation, wash extensively the top of the gels with deionized water to remove any remaining 2-butanol. Remove the excess of water by suction with a syringe.
3. After the transfer of the capillary gel on top of the gradient polyacrylamide gel (see **Subheading 3.2.**, **step 6**), fill the upper and the lower reservoirs with the running buffer and apply a constant current of 40 mA per gel. The separation usually requires 5 h. The temperature in the lower tank buffer is maintained at 10°C during the run.
4. At the end of the run, turn off the power, rinse briefly the gel in distilled water for a few seconds and process for gel staining. A typical gel pattern of plasma proteins stained with the ammoniacal silver nitrate method is shown in **Fig. 2** ***(6, 7).***

4. Notes

1. The addition of thiosulfate to the gel delays the appearance of background staining with the ammoniacal silver nitrate method ***(6, 7).***
2. Human plasma proteins have been efficiently separated with the following sample preparation: 5 µL of plasma (containing approximately 400 µg

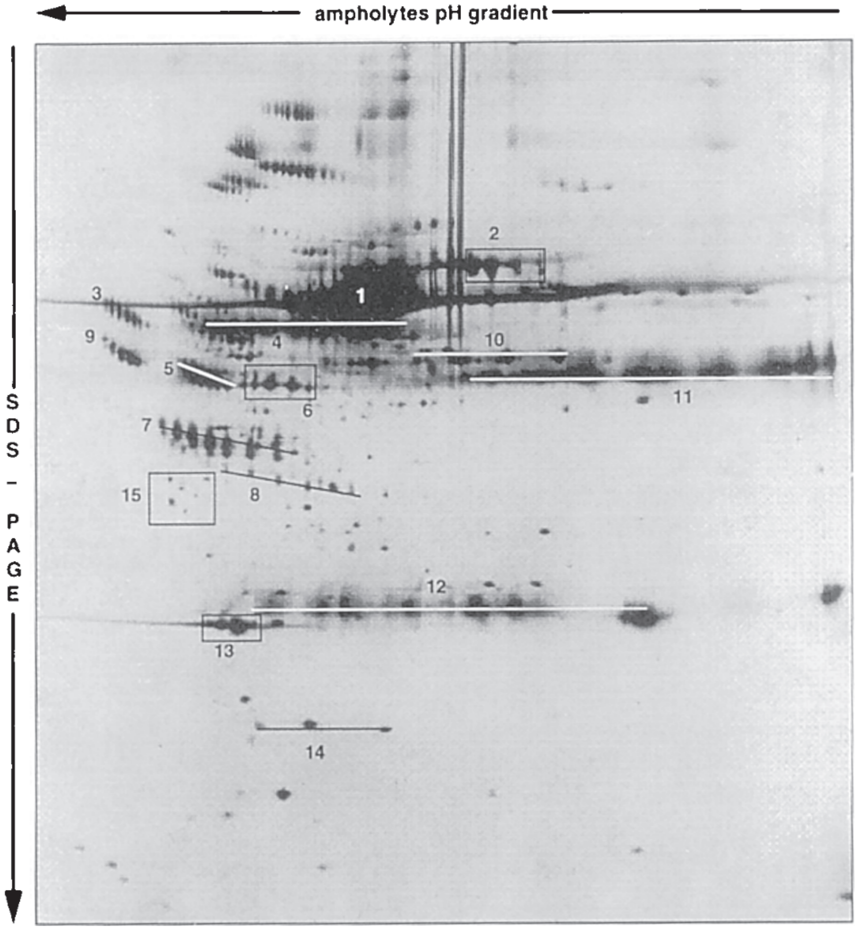

Fig. 2. Silver stained plasma proteins separated by two-dimensional polyacrylamide gel electrophoresis using carrier ampholyte pH gradient in the first dimension. *1,* albumin; *2,* transferrin; *3,* α_1-antichymotrypsin; *4,* IgA α-chain; *5,* α_1-antitrypsin; *6,* fibrinogen γ-chain; *7,* haptoglobin β-chain; *8,* haptoglobin cleaved β-chain; *9,* α_2-HS-glycoprotein; *10,* fibrinogen β-chain; *11,* IgG γ-chain; *12,* Ig light chain; *13,* Apolipoprotein A-1; *14,* haptoglobin α_2-chain; *15,* apolipoprotein J.

of proteins) are solubilized in 10 µL of lysis solution A (SDS-DTE) and heated in boiling water for 5 min. After cooling for 2 min at room temperature, 485 µL of lysis solution B is added. For silver stained gel, 30 to 40 µL of the final diluted sample is loaded on the top (cathodic side) of the capillary gel.

3. The best separation of complex protein mixtures is performed when less than 100 µg of proteins are loaded onto the capillary gel. Overloading may

cause streaking and inadequate resolution of spots. The use of a highly sensitive staining method (silver staining) allow to applied a low amount of proteins onto the IEF gel. For silver stained gel, concentrations up to 25–40 µg per gel are enough.

4. During the sample loading, it is very important to avoid the formation of air bubbles between the gel and the sample. After the loading, the Hamilton syringe should be withdrawn slowly along the side wall.
5. If more than 20 kVh is applied during the isoelectic focusing, more cathodic drift occurs and the protein pattern is not stationary.
6. Low pressure on the 1 ml syringe should be exerted to extrude the capillary gel. If too much pressure is applied, small lumps will be formed on the gel.
7. Precise determination of the volume of solution necessary to cast the gels should first be done by measuring the volume of distilled water required to fill one resolving gel or the casting chamber.

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Vertical Agarose Electrophoresis and Electroblotting of High-Molecular-Weight Proteins

Marion L. Greaser and Chad M. Warren

1. Introduction

Very large molecular weight proteins are difficult to separate by electrophoresis because of their poor penetration into gels using the Laemmli SDS polyacrylamide system ***(1).*** Protein migration in SDS gels has been found to be linear with the log of the molecular weight ***(2),*** so the larger the protein, the more poorly it is resolved from other big proteins. Many workers have attempted to solve this problem by using very low concentration acrylamide gels ***(3),*** acrylamide mixed with agarose ***(4),*** or acrylamide gradients ***(5)*** to better separate large proteins. Low concentration acrylamide gels are difficult to use because of their mechanical fragility and distortion during handling; these problems are magnified when blotting is attempted. An additional difficulty in blotting very large proteins is their poor transfer to the membrane. Inclusion of 2-mercaptoethanol in the transfer buffer improves transfer efficiency, but acrylamide gels stained after transfer typically still contain most of the giant muscle protein titin ***(6).***

A new electrophoresis system using SDS agarose for protein electrophoresis and blotting has been described ***(7).*** An example showing the resolution for several muscle samples containing large proteins is shown in **Fig. 1.** Migration distance shows a linear relationship with the log of the molecular weight ***(7)***. This system allows quantitative transfer of proteins from the gel with much higher reproducibility than can be achieved with methods using low percentage acrylamide.

2. Materials

2.1. Apparatus

1. SE 600 Slab Gel Unit with 16 × 18 cm glass plates (Hoefer) or a similar commercial gel unit (*see* **Note 1**).

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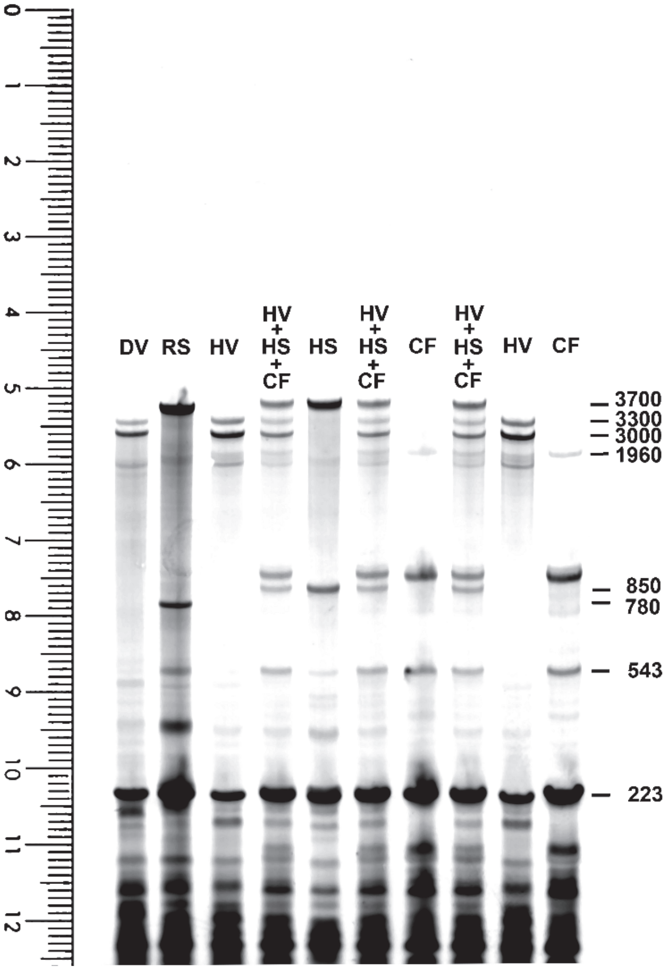
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HV + HS + CF
HV
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3700
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3000
1960
850
780
543
223

Fig. 1. SDS 1% agarose gel stained with silver. A centimeter ruler is shown on the left and the sizes of the various protein bands in KDa are listed on the right. DV, dog ventricle; RS, rat soleus; HV, human ventricle; HS, human soleus; CF, crayfish claw muscle. Human soleus titin is 3700 KDa and human ventricle has two titin bands of 3300 and 3000 KDa. The bands at 780 and 850 KDa are rat and human nebulin respectively. The myosin heavy chain is 223 KDa. Blotting proteins this size from acrylamide gels usually results in incomplete transfer, but full transfer can be achieved with agarose *(7).*

2. 65°C oven.
3. A constant current power supply.
4. Circulating cooler.
5. TE62 Tank Blotting Unit, Hoefer.

2.2. Reagents

1. Acrylamide gel for plug; 38.5 % acrylamide. Weight 37.5 g of acrylamide (Biorad) and 1 g DATD (Biorad) into a beaker, add about 50 ml of water, stir till dissolved, dilute to 100 ml. Filter through a 0.45 micron filter (such as a Millex-HA, Millipore Corporation). Store in a brown bottle in the cold room (4°C). Danger! Avoid skin contact.
2. Reservoir and agarose gel buffer concentrate (5X); 0.25 *M* Tris, 1.92 *M* glycine, 0.5% SDS. Store at room temperature.
3. Ammonium persulfate; Prepare a 100 mg/ml solution in water; store frozen in 0.5 ml aliquots (stable indefinitely at −20°C).
4. Sample buffer; 8 *M* urea, 2 *M* thiourea, 0.05 *M* Tris-HCl (pH 6.8), 75 mM DTT, 3% SDS, 0.05% bromophenol blue (adapted from ***8***). (Dissolve urea and thiourea and treat with mixed bed resin to remove ionic constituents; then add remaining ingredients. Store at −20 °C).
5. 50% v/v glycerol.
6. Transfer buffer; 20 m*M* Tris, 150 m*M* glycine, 20% v/v methanol ***(7, 8)*** or 10 m*M* CAPS, pH 11 (Sigma Chemical Company) ***(9).*** For high molecular weight proteins, add SDS and 2-mercaptoethanol to 0.1% and 10 m*M* respectively to the transfer buffer.
7. SeaKem Gold agarose (Lonza Group Ltd.)

3. Methods

3.1. Gel Preparation

1. Volumes listed will provide enough solution for two 16 × 18 cm gels with 1.5 mm spacers. One is used for staining either with Coomassie blue or with silver with a special procedure for agarose ***(7),*** the other for blotting.
2. Clean plates and spacers with soap, rinse with distilled water and finally with ethanol.
3. Assemble gel plates. Place plate on clean bench top. Place spacers hanging half the way off each side of plate. Place second plate on top. Stand up plates and place one side into the clamp. Align spacer with side of plates and clamp and push spacer down so that bottom is flush with the glass plates (top buffer will leak if spacers are not flush with plates).
4. Pour acrylamide plugs in bottom of gel plate assembly (*see* **Note 2**): In a 15 ml plastic beaker add: 1.924 ml de-ionized water, 1.7 ml 50% glycerol, 2.12 ml 3 *M* Tris (pH 9.3), 2.72 ml acrylamide (40%), 24 µL 10% ammonium persulfate, and 13 µL TEMED. (*see* **Note 2**) Mix by pipeting a few times. Immediately add 2.5 ml to each gel assembly. Add a small amount of water on top of each plug to level the upper surface and provide an oxygen barrier. Allow gel to polymerize for 20–30 minutes. Drain off water layer by inverting gel plate assembly on a paper towel.
5. Place assembly, 20 lane sample combs, and 60 ml plastic syringe in a 65°C oven for 10 minutes (*see* **Note 3**).

6. Weigh 0.8 g of SeaKem Gold agarose (*see* **Note 4**) into a 600 ml beaker (*see* **Note 5**). To a 100 ml graduated cylinder add 48 ml of 50% v/v glycerol (*see* **Note 6**), 16 ml 5X electrophoresis buffer, and bring volume up to 80 ml with deionized water. Place parafilm over top of the graduated cylinder, mix by inverting a few times, and pour solution into the 600 ml beaker containing the agarose. Place saran wrap over top of beaker and poke a few holes in the saran wrap. Weigh beaker with contents. Place beaker in a microwave oven along with a separate beaker of de-ionized water. Heat for a total of 2 minutes (stop every 30 seconds to swirl – protect hand with an insulated glove) (*see* **Note 7**).
7. Allow agarose to cool for a few minutes at room temperature. Reweigh, and add sufficient heated de-ionized water to replace that lost by evaporation.
8. Draw up about 40 ml of agarose in the prewarmed 60 ml Luer-Lock syringe and pour each gel slowly until it just overflows the top of the plates. Try to avoid formation of bubbles (if bubbles present, bring them to the top of the gel and pinch them with the sample comb). Insert sample combs and allow unit to cool at room temperature for about 45 minutes (*see* **Note 8**),

3.2. Electrophoresis Setup and Sample Loading

1. Add 4 liters of buffer to lower chamber (3200 ml de-ionized water plus 800 ml 5X electrophoresis buffer). Start cooling unit and stir bar (gels run at 6°C).
2. Prepare 600 ml upper chamber electrophoresis buffer (same concentration as lower chamber buffer). Add 2-mercaptoethanol (final concentration of 10 m*M*). Buffer will be poured into top chamber after samples are loaded and assembly placed in unit.
3. Take combs out of gels by bending them back and forth to detach from gel and slowly pull them up. Pour a small amount of upper chamber buffer into a 15 ml beaker and pipette buffer into first and last wells (the rest will fill over). Add buffer to remove any trapped bubbles. Insert pipette tip to deposit sample in bottom of the sample well. Skip the first and last lanes (*see* **Note 9**).
4. Running gels. Once samples are loaded, put upper chamber on the assembly. Pour upper chamber buffer into upper chamber from corners (do not pour buffer directly over wells). Place lid on unit, and connect to power supply. Turn electrophoresis unit on and run at 30 mAmps (2 gels) for 3 hours.

3.3. Staining and Western Blotting

1. After tracking dye reaches the bottom of the acrylamide plug, turn off the power and disassemble the plates. Cut off sample wells and acrylamide plug and discard. Soak the remaining agarose gel in 10 m*M* CHAPS (pH 11.0), 0.1% SDS, and 10 m*M* 2-mercaptoethanol for 30 minutes with gentle shaking.
2. The gel is then placed on top of either a sheet of PVDF or nitrocellulose, assembled into the transfer unit, and the protein electrophoretically transferred using 40 V constant voltage for 2–3 hours (*see* **Note 10**).
3. Blotted proteins can then be treated using conventional procedures with either colorimetric (horseradish peroxidase or alkaline phosphatase substrates) or ECL (enhanced chemiluminescence) methods.

4. Notes

1. The agarose gel procedure works equally well with small format gels (i.e., 8 × 10 cm).
2. The acrylamide plug is used to prevent the agarose from slipping out of the vertical gel plate assembly. Use of DATD as the cross-linker provides a stickier bond of the acrylamide to the glass plates than if a conventional bisacrylamide cross-linker is used. Plugs can be poured a day before making the gel (place tape or parafilm over the top of the plates to prevent drying and store in cold room).
3. Preheating the glass plate assembly, well comb, and syringe prevents premature agarose gelling when the solution touches the colder surfaces. In addition the plates are less likely to crack during pouring if they are closer to the temperature of the hot agarose.
4. The supplier for SeaKem Gold agarose has changed twice since 2003. Biowhittaker was succeeded by Cambrex who was followed by Lonza Group Ltd, Muenchensteinerstrasse 38, CH-4002 Basel, Switzerland.
5. It is essential to use SeaKem Gold agarose for optimal migration of high molecular weight proteins. This type has large pore size and excellent mechanical stability. Other types of agarose may be used, but the protein mobility will be significantly reduced.
6. Glycerol is included in the mixture to increase the solution viscosity inside the gel and thus sharper protein bands.
7. Periodic swirling during the heating step eliminates non-hydrated agarose granules in the final gel.
8. Sample combs should extend no longer than 1 cm into agarose; otherwise they may be difficult to remove. Gels can be used right away or stored overnight in cold room.
9. Conventional sample buffers may not be dense enough for the sample to stay at the bottom of the well. If necessary add additional glycerol (up to 30% v/v final concentration) to increase sample density.
10. The disulfide bond formation of large proteins during electrophoresis also retards their migration out of the gel onto blots during transfer. Thus inclusion of 2-mercaptoethanol in the transfer buffer improves efficiency of transfer of high molecular weight proteins. The use of the agarose electrophoresis system with inclusion of 2-mercaptoethanol in the transfer buffer results in complete transfer of all high molecular weight proteins out the gel, including titin (Mr 3000 to 3700 KDa subunit size) ***(7).***

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Two-Dimensional PAGE of High-Molecular-Mass Proteins

Masamichi Oh-Ishi and Tadakazu Maeda

1. Introduction

Many high-molecular-mass proteins (MW>100 kDa) are known to be involved in cytoskeleton, defense and immunity, transcription and translation in higher eukaryotic organisms. Though a variety of protein separation techniques have been described, at the moment purification of a high-molecular-mass protein remains a difficult task. O'Farrell ***(1)*** was the first to devise a two-dimensional gel electrophoresis (2-DE) technique which could detect more than 1000 spots in a gel. Though it is evident that this method was quite powerful in his days, the method is not particularly suitable in analyzing high-molecular-mass proteins larger than 200 kDa. When skeletal muscle, for example, is simultaneously analyzed by sodium dodecyl sulfite (SDS)-polyacrylamide gel electrophoresis (PAGE) and O'Farrell's 2-DE method, myosin heavy chain (approx 200 kDa) is clearly seen in the former gel, but not in the latter. Hirabayashi ***(2)*** was the first to develop a 2-DE method which could analyze high-molecular-mass proteins, including myosin heavy chain and dystrophin, as large as 500 kDa ***(3).*** His trick was the use of agarose gel instead of polyacrylamide gel for the first-dimensional isoelectric focusing (IEF). Agarose gel, when used for IEF, can analyze much larger proteins than the polyacrylamide gel can. Oh-Ishi and Hirabayashi ***(4)*** further improved the method by adding 1 *M* thiourea and 5 *M* urea in an agarose IEF medium. Thiourea is a potent protein solubilizing reagent especially effective for high-molecular-mass proteins that could enter the first-dimensional agarose IEF gel. Here, we describe the 2-DE method with agarose gels in the first dimension (agarose 2-DE) that is compatible with analyzing high-molecular-mass proteins.

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2. Materials

2.1. Equipment

1. Apparatus for first-dimensional agarose IEF (AE-6300 electrophoresis unit) ATTO (Tokyo, Japan).
2. Glass tubes (260 mm × 3.4 mm ID) for agarose IEF (ATTO).
3. Dialysis membranes.
4. Rubber bands.
5. Syringe to fit thin 30-cm-long polyethylene tubing.
6. Electrophoresis power supply (Crosspower 3500) (ATTO).
7. Horizontal electrophoresis apparatus (Multiphor II, GE Healthcare).
8. Immobiline DryStrips pH 3–10 L (GE Healthcare).
9. Perista pump (SJ-1211) (ATTO).
10. Glass tubes (300 mm × 5 mm ID) for protein fixation on agarose IEF gels.
11. Polyvinyl chloride (PVC) tubing (400 mm × 2 mm ID).
12. Vertical type apparatus for second-dimensional SDS-PAGE (NA-1200 electrophoresis unit) Nihon Eido (Tokyo, Japan).
13. Glass plates with a 1.5-mm thick frame (size 165 mm × 240 mm) for second-dimensional SDS-PAGE (Nihon Eido).
14. Plain glass plates (size 165 mm × 240 mm) for second-dimensional SDS-PAGE (Nihon Eido).

2.2. Reagents

1. Agarose IEF gels: Agarose IEF and pharmalyte were purchased from GE Healthcare. D-sorbitol, urea, and thiourea were from Wako Pure Chemicals (Osaka, Japan). Agarose IEF gels were prepared according to the protocols in section 3.1.1 Gel preparation and in **Table 1**.
2. Extraction medium: 7 *M* urea, 2 *M* thiourea, 2% CHAPS, 0.1 *M* dithiothreitol (DTT), 2.5% Pharmalyte, and protease inhibitors (Complete Mini ethylenediaminetetraacetic acid [EDTA]-free; Roche).
3. Overlaying solution: 4 *M* urea, 1 *M* thiourea.
4. Cathode buffer: 0.2 *M* NaOH.
5. Anode buffer: 0.04 *M* DL-aspartic acid.
6. Protein fixing solution: 10% trichloroacetic acid, and 5% sulfosalycylic acid.
7. Incubation medium: 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue, and 0.05 *M* Tris-HCl, pH 6.5.
8. Staining solution: 0.02% PhastGel Blue R (coomassie brilliant blue R-350; GE Healthcare), 30% methanol, and 10% acetic acid.
9. Destaining solution: 30% methanol, and 10% acetic acid.

3. Methods

3.1. Agarose Gels for IEF

3.1.1. Gel preparation

1. We prepared the first-dimensional agarose IEF gels following the same procedure as described in our former report *(5),* to which we added the modifications described

here. Three agarose IEF gels (180 mm in length and 3.4 mm in diameter) containing 1 *M* thiourea and 6 *M* urea were prepared by the present protocol.

2. Agarose IEF (0.10 g) and D-sorbitol (1.2 g) were put into a 50 mL beaker, and dissolved in 5.8 mL distilled water at room temperature (solution A) (*see* **Note 1**).
3. Solution A was boiled 10 times for 15 s in a microwave oven until the solution became clear, and was kept in a 70°C water bath for 5 min.
4. A mixture of urea (3.6 g) and thiourea (0.76 g) powder was put into the solution A at 70°C, which we shall call solution B hereafter (*see* **Note 2** and **Table 1**). The volume of solution B was then adjusted to 9.0 mL with distilled water, and was kept at room temperature.
5. Solution B was divided into three test tubes: 1.0 mL for acidic, 4.5 mL for neutral, and 2.5 mL for basic solutions.
6. Four kinds of Pharmalyte (pH 2.5–5 for acidic, pH 3–10 and pH 4–6.5 for neutral and pH 8–10.5 for basic solutions) were added to the respective tubes according to the protocol shown in **Table 1**.
7. A glass tube (standard size: 260 mm × 3.4 mm ID) was prepared beforehand, the bottom of which was covered with a piece of dialysis membrane and tied with a rubber band.
8. The glass tube was set to AE-6300 electrophoresis unit (apparatus for agarose IEF produced by ATTO).
9. The acidic, neutral, and basic solutions were sucked in with one syringe each through thin 30-cm-long polyethylene tubing. First, the acidic solution was softly injected into the glass tube until the meniscus reached a height of 20 mm from

Table 1
Protocols for Making Agarose Isoelectric Focusing (IEF) gels

	Agarose IEF	0.10 g	
	D-sorbitol	1.20 g	
	Distilled water	5.80 mL	
	Dissolved at 100°C——> Solution A		
	————Set at 70°C————-		
	Urea	3.60 g	
	Thiourea	0.76 g	
	Dissolved at 50°C——> Solution B		
	Addition of distilled water until solution B comes to 9.0 mL		
	————Set at room temperature————		
Solution B	1.0 mL	4.5 mL	2.5 mL
Pharmalyte	↓	↓	↓
pH 2.5–5	100 μL	—	—
pH 3–10	—	300 μL	—
pH 4–6.5	—	150 μL	—
pH 8–10.5	—	—	250 μL
	↓	↓	↓
	Acidic solution	Neutral solution	Basic solution

the bottom of the tube. Next, the neutral solution was slowly injected until the meniscus reached a height of 135 mm from the bottom, and then the basic solution was carefully injected until the meniscus was 180 mm from the bottom. A 10 μL overlaying solution was gently layered on top of the agarose solution, which made it easier for proteins to enter the agarose IEF gel. The glass tube was filled with the chamber and kept there at least 6 h, until the agarose solution gelled (*see* **Note 3**).

3.1.2. Sample Preparation

1. Freshly obtained mammalian tissues were cut into small blocks, quickly frozen in liquid nitrogen, and stored at −80°C until use. Each frozen tissue piece (about 10 mg) was homogenized with a Teflon glass homogenizer in an extraction medium (20 times the volume of the tissue pieces) (e.g. brain, muscle, kidney, and liver). Homogenates of the tissues were centrifuged at 112,000 g for 20 min, and the clear supernatant was subjected to the agarose IEF gel.

3.1.3. Sample Application

1. We added 10–200 μL of protein sample solution at the cathodic end of the gel, and gently filled the overlaying solution above the sample solution to the top of the glass tube. We added anode buffer to the lower reservoir, and cathode buffer to the upper reservoir. First-dimensional IEF was conducted at 600 V for 18 h at 4°C.
2. Then the agarose gel was transferred onto the top of the second-dimensional SDS gel, either directly or after proteins in the gel were fixed (*see* **Note 4**).

3.2. Second-Dimensional SDS-PAGE

1. Slab gels for second-dimensional electrophoresis were 12% polyacrylamide gels (200 mm × 120 mm × 1.5 mm). Second-dimensional SDS-PAGE was carried out according to the stacking system of Laemmli ***(6)*** with the slight modification of adding 1% SDS both in the stacking and separation gels.
2. The first-dimensional agarose IEF gel was loaded on top of the stacking gel without SDS equilibration (*see* **Note 5**) and covered with 1% agar solution to keep the agarose IEF gel in place. An incubation medium was overlaid on the IEF gels.
3. The second-dimensional gel electrophoresis was started with a constant current at 40 mA for 1 h and continued at 70 mA until the end of the run.
4. The slab gels were first soaked and shaken overnight in a destaining solution, which removed Pharmalytes from the gel (*see* **Note 6**). The slab gels were then stained with a staining solution containing PhastGel Blue R and destained with the destaining solution.

3.3. Comparison of IPG-Dalt with Agarose 2-DE

1. In this study, we compared the agarose 2-DE system with IPG-Dalt (immobilized pH gradients for IEF in the first dimension, SDS-PAGE in the second dimension) to assess their capability of analyzing high-molecular-mass proteins.
2. The first-dimensional IEF with IPGs was performed essentially as described by Gorg *et al*. ***(7)***. IPG dry strips (Immobiline DryStrips pH 3–10 L) and Multiphor II (GE Healthcare) were used in this study.

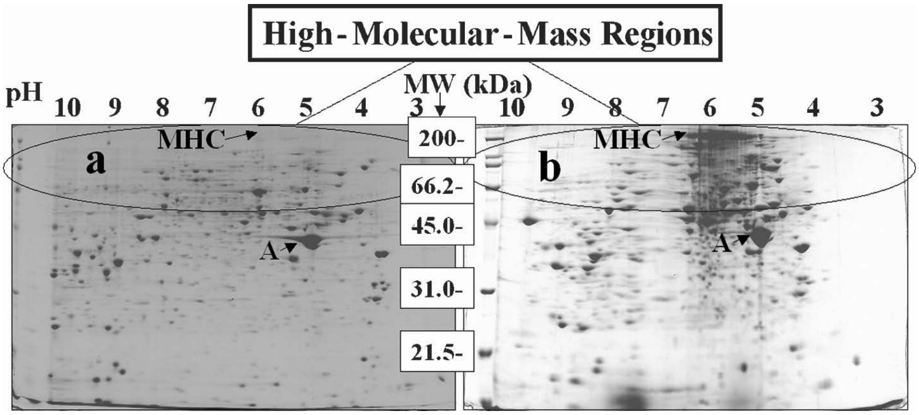

Fig.1. Comparison of IPG-Dalt and agarose two-dimensional gel electrophoresis (2-DE). Tissues used are rat duodenum. **(A)** A 2-DE gel with immobilized pH gradients (IPGs) in the first dimension. **(B)** A 2-DE gel with agarose gels in the first dimension. Proteins loaded on an agarose isoelectric focusing gel and an IPG gel were 740 µg. Note that spot densities are different in the high-molecular-mass regions. A, actin; MHC, myosin heavy chain. The gels were stained with PhastGel Blue R.

3. Coomassie-stained 2-DE patterns of rat duodenum (**Fig. 1**) revealed that high-molecular-mass proteins larger than 150 kDa could be analyzed with the agarose 2-DE but not with IPG-Dalt.

4. Notes

1. Shake the beaker gently until a mixture of agarose IEF and D-sorbitol powder is completely suspended.
2. Immediately after the mixture of urea and thiourea powder was put into the beaker, solution B was stirred with a magnetic stirrer until the urea and thiourea were completely dissolved.
3. As a side effect of thiourea, a thiourea-urea agarose IEF solution does not gel at room temperature but at 4°C, and the gel formed at 4°C does not melt even when the gel is returned to room temperature. From the practical point of view of an experimental scientist, the 1 *M* thiourea/5 *M* urea-agarose IEF gel was a tremendous improvement over the 7 *M* agarose IEF gel originally used ***(2)***, because the agarose solution temperature no longer needed to be kept above 40°C when preparing agarose IEF gels.
4. When proteins in the agarose gel were fixed prior to the second dimensional electrophoresis, the gel was extruded into a 300-mm-long, 5-mm-diameter glass tube filled with a protein fixing solution containing 10% trichloroacetic acid and 5% sulfosalycylic acid. Both ends of the 5 mm diameter tube were connected to a Perista pump with PVC tubing to form a closed

circuit filled with the fixing solution. When more than two gels were to be fixed, each of the gels was respectively extruded in a 5-mm-diameter glass tube, which was serially connected to one another to form a bigger closed circuit filled with the fixing solution. The proteins in the gel were fixed by 1 h circulation of the fixing solution in the 5-mm-diameter glass tube with a Perista pump, followed by 1 h circulation of 500 mL distilled water.

5. Avoid SDS equilibration step because the step easily spreads proteins out of the agarose gel.
6. Thorough removal of Pharmalytes from first-dimensional agarose IEF gel is useful in two respects: one is to reduce spot deformations by Pharmalyte pH 8–10.5 in the basic pI (>8) and low molecular mass (<30 kDa) region of a 2-DE gel and the other is to lower the background level of a Coomassie-stained 2-DE gel.

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Casting Immobilized pH Gradients

Elisabetta Gianazza

1. Introduction

One of the main requirements for a 2-D protocol is reproducibility of spot position, and, indeed, the technique of isoelectric focusing on immobilized pH gradients (IPGs) is ideally suited to provide highly reproducible 1-d separations. IPGs are obtained through the copolymerization of acidic and basic acrylamido derivatives of different pKs within a polyacrylamide matrix ***(1, 2)*** **(Fig. 1)**. The pH gradient may be devised by computer modeling either with a linear or with an exponential course. IPGs are cast from two limiting solutions containing the buffering chemicals at concentrations adjusted to give the required pH course upon linear mixing. For consistent results, gradient pouring and polymerization are carried out under controlled conditions. The covalent nature of the chemical bonds formed during the polymerization step results in a permanent stability of the pH gradient within the matrix. Conflicting requirements during the focusing procedure prevent any effective use of IPGs into capillary tubes ***(3)***: The need to buffer with carrier ampholytes (CAs) the pH extremes caused by the migration of the polymerization catalysts is contrasted by the adverse effects of the electroendosmotic flow brought about by the addition of CAs to the gel phase. The demand for the IPG gels to be backed by a binding support—they are usually cast on GelBond™ foils—results in dimensional stability between 1-D and 2-D as a further assistance to reproducibility.

Toward the aim of reproducibility, batch production and quality control as allowed by an industrial process give the commercially available IPG strips (e.g. Immobiline DryStrip™ from GE-Amersham, Uppsala, Sweden) obvious advantages over homemade slabs. Moreover, the pH course of the commercial product is being carefully characterized in chemicophysical terms, by assessing the dissociation constants of the acrylamido buffers under the experimental

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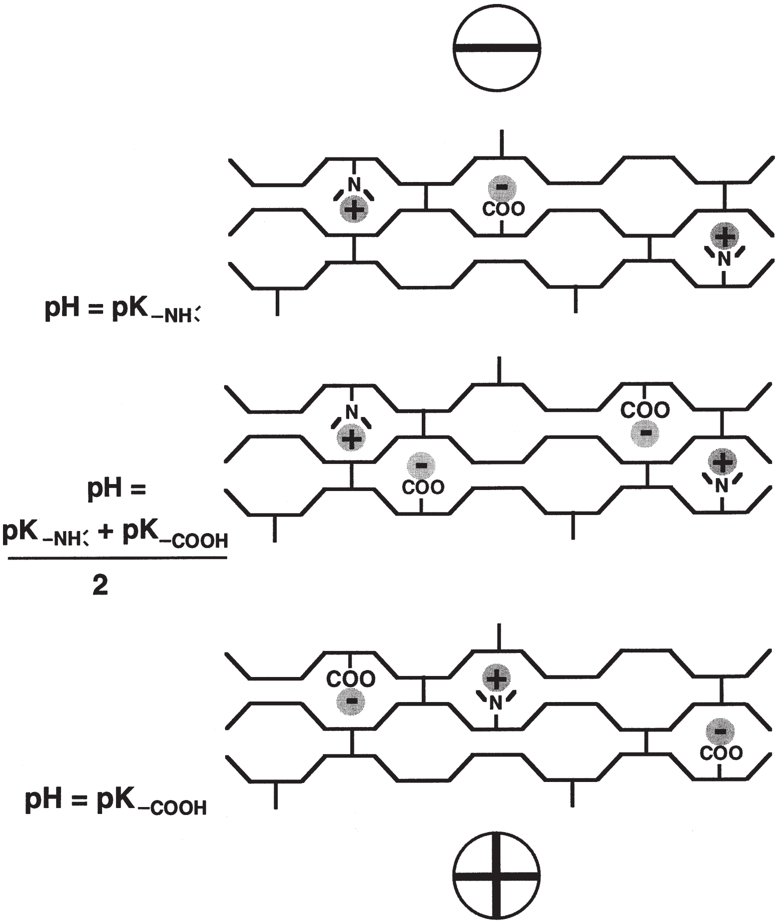


Fig. 1. Structure of the IPG matrix. Different regions of an IPG made up with a basic and an acidic Immobiline are schematically drawn, from cathode to anode. In each region, pH depends from the concentration ratio between the basic and the acidic dissociating groups. From the Henderson-Hasselbalch equation, where the concentration of the basic monomer is twice the concentration of the acidic monomer, pH equals the pK of the base, where the concentration of the basic monomer is one half the concentration of the acidic monomer pH equals pK of the acid, where the two concentrations are alike pH equals the mean between the two pKs.

conditions relevant to the 1-d run of an IPG-DALT *(4)*. The aim of this effort is to connect focusing position reliably with p*I* *(5)*; any discrepancy between computed and experimental values for known proteins would then hint at the occurrence of posttranslational modifications *(6)*.

From the above it seems this chapter could shrink to the statement: Use the readymade IPG strips according to the manufacturer's instructions. However, there are reasons, and not only of economical order, for laboratories to cast their own slabs. The main one is the variability of the analytical needs. 2-D protocols usually aim at the resolution of all peptide components of a complex mixture. Although the proteins in cellular extracts and biological fluids have mostly mildly acidic p*I* values, this is not true of all samples, and the optimal pH course should be devised accordingly. Moreover, after specific qualitative or quantitative variations have been detected for some spots as the result of a given experimental treatment, it is usually worth investigating the area of interest under conditions of maximal resolution. This is especially true if the spots have to be identified or characterized (e.g., by mass spectrometry [MS] techniques) ***(7)***. IPGs allow the tailoring of wide, narrow, or ultranarrow pH gradients, whereas migration on continuous or gradient PAA slabs of different %T in 2-D may further improve resolution of the spots of interest. In order to optimize reproducibility and to increase throughput, laboratories handling a very large number of gels may consider a medium-scale production with gradient pouring through mechanical devices (computer-driven burettes) **(8, 9)**. In any event, even at the typical laboratory level, the procedure of IPG casting is reliable and allows a remarkable reproducibility of results ***(10)***. The following will try to convince the readers that it is also an easy one.

2. Materials

2.1. Equipment

1. Polymerization cassette:
   a. A molding plate with a 0.5-mm-thick permanent frame. Available in two sizes, either 12.5 *x* 25 cm, w *x* h (cat. no. C2418, from Sigma, St. Louis), or 25 *x* 12.5 cm, w *x* h (cat. no.80-1106-89, from GE-Amersham).
   b. Gel-supporting plate.
   c. Gel-binding foil, e.g., GelBond PAG™ (GE-Amersham, Sigma).
   d. Clamps.
2. Gradient-mixing device:
   a. A two-vessel chamber (e.g., gradient maker cat. no. Z340405 or Z340413, from Sigma).
   b. Magnetic bars.
   c. Outlet to the mold, either a one-way silicone tubing (od 3 mm, id 1.5 mm, ca. 12 cm long, equipped with a 2-cm teflon tip) or multiple inlets cast by adapting the tips, ca. 6 cm long, of butterfly needles gauge 21 to a T-connector, available from Cole-Parmer (cat. no. K6365–70).
   d. A stirrer providing constant and even operation at medium to low rpm rating with minimal overheating.
   e. Screw-jack rising table.
3. Electrophoresis apparatus:

for upside-up (IPG matrix up) runs:

   a. Horizontal electrophoretic chamber with movable electrodes.
   b. Power supply able to deliver >3000 V. We recommend using a power supply with an amperometer sensitive to <1-mA currents.
   c. Thermostating unit.

   for upside-down (IPG matrix down) runs:

   a. Specialized IEF cells (e.g., IPG-Phor from GE-Amersham)

4. Forced-ventilation oven at 50°C.
5. Shaking platform.
6. Fan.

2.2. Reagents

1. Acrylamido buffers: 0.2-*M* solutions (*see* **Table 1**), prepared either in water with 5 ppm of hydroquinone methylether as polymerization inhibitor for the acidic monomers or in *n*-propanol for the basic monomers. The chemicals are available from GE-Amersham (as Immobiline™) or from Fluka (Buchs, Switzerland). Storage at 4 °C; the expiration date is given by the manufacturer. The products are defined as irritant, whereas *n*-propanol is classified as highly flammable.
2. Acrylamide monomers stock solution: 30% T, 4% C ***(11)***. Dissolve in water 1.2 g *bis*acrylamide and 28.8 g acrylamide/100 mL final volume. Filter the stock through 0.8-mm membranes and store at 4°C for about 2 mo. The chemicals should be of the highest purity. Acrylamide has been recognized as a neurotoxin.
3. 40% w/v Ammonium persulfate solution: Dissolve 200 mg of ammonium persulfate in 440 µL of water. Store up to a week at 4°C.
4. TEMED: Store at 4°C for several months.
5. 87% v/v Glycerol.
6. 1 *M* Acetic acid: Dilute 57.5 mL of glacial acetic acid to 1 L.
7. Dimethyldichlorosilane solution: 2% v/v solution of dimethyldichlorosilane in 1,1,1-trichloroethane. Both chemicals are available from Merck (Darmstadt, Germany), or a ready-made solution is marketed by GE-Amersham as RepelSilane™.
8. Glycerol washing solution: 1% v/v glycerol.

Table 1
pK values of Immobilines™

Chemicals	water solution, 25°[a]	PAA gel, 10°C[a]	8 M urea in PAA gel, 10°C[b]
Immobiline pK 3.6™	3.58	3.57	4.47
Immobiline pK 4.6™	4.61	4.51	5.31
Immobiline pK 6.2™	6.23	6.21	6.71
Immobiline pK 7.0™	6.96	7.06	7.53
Immobiline pK 8.5™	8.52	8.50	8.87
Immobiline pK 9.3™	9.27	9.59	9.94

[a] from LKB Application Note 324; [b] from ***(29)***

3. Method

3.1. Selecting a pH Range and a Gradient Course

The formulations for narrow to wide-range IPGs have been reported in a series of papers ***(12–15)***, and the recipes for casting the gradients collected ***(2)***. Most of the formulations use the acrylamido buffers marketed as Immobiline™ by GE-Amersham, since the pK distribution in their set is even and broad enough for all applications in the 4.0–10.0 pH region. For specific purposes—increased hydrophilicity, narrow ranges, pH extremes—other chemicals are available as acrylamido derivatives ***(16)*** or have been custom-synthesized ***(17)***. These chemicals are available from Fluka. Computer programs for pH gradient modeling, with routines optimizing the concentrations of the acrylamido buffers required in the two limiting solutions in order to give the expected pH course, have been described in the literature ***(18–20)***. For most practical applications, a wide pH gradient is required for 2-D mapping in order to resolve all components of a complex biological sample. A statistical examination of the p*I* distribution across characterized proteins as well as the typical maps of most biological specimens show a prevalence of acidic to mildly alkaline values over basic proteins. A nonlinear pH course is then expected to give improved resolution of most proteins in a complex sample. An exponential gradient whose slope increases from pH 6.0 to 7.0 and to 8.0 optimally resolves most samples run in IPGs: one such recipe ***(14)*** is given in **Table 2** (I), whereas

Table 2
IPG 4–10, with a non-linear course[a]

	Recipes for 5.1 + 5.1 mL gradients							
	I: Whole range		II: Acidic region		III: Neutral region		IV: Basic region	
Chemicals	A[b] µL	B[c] µL	A µL	B µL	A µL	B µL	A µL	B µL
pK 0.8	135	16	135	62	103	45	86	14
pK 4.6	130	–	130	52	98	33	78	–
pK 6.2	153	46	153	89	126	73	110	46
pK 7.0	–	61	–	37	15	46	24	61
pK 8.5	–	23	–	14	6	17	9	23
pK 9.3	–	43	–	26	11	32	17	43
1*M* acetic acid	–	14	–	8.4	3.5	10.5	5.6	14
T30 C4	700	700	700	700	700	700	700	700
87% Glycerol	1000	–	1000	–	1000	–	1000	–
TEMED	3.1	2.7	3.1	2.9	3	2.8	2.9	2.7
40% APS[d]	4.75	4.75	4.75	4.75	4.75	4.75	4.75	4.75
pH at 25°C[e]	4.13	9.61	4.13	6.27	4.67	7.07	5.25	9.61

[a] from ***(14)***; pH gradient course in Figure 2; [b] acidic solution; [c] basic solution; [d] to be added to the solutions after transferring to the gradient mixer and starting the stirrer; [e] pH of the limiting solutions

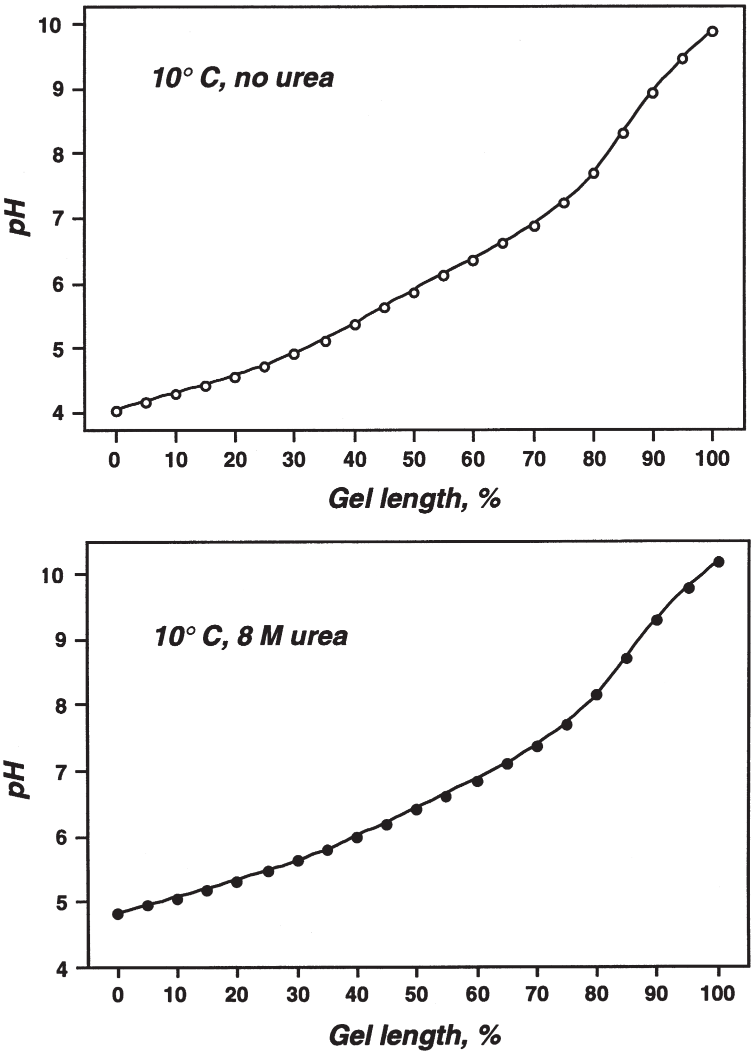


Fig. 2. Course of the gradient for the 4–10 nonlinear IPG. The top panel shows the pH course computed for the gradient in **Table 2** (*14*) after grafting into a PAA matrix (pK values from **Table 1,** 2nd column); the bottom panel corresponds to the pH values corrected for the presence of 8 *M* urea (pK values from **Table 1,** 3rd column).

the gradient is plotted in **Fig. 2** (notice that the course of this gradient *differs* from commercial 4–10 NL as having a much flatter acidic region).

The narrow ranges in **Table 2** (II–IV) have been interpolated from the sigmoidal gradient above, and provide a better resolution of the acidic, neutral,

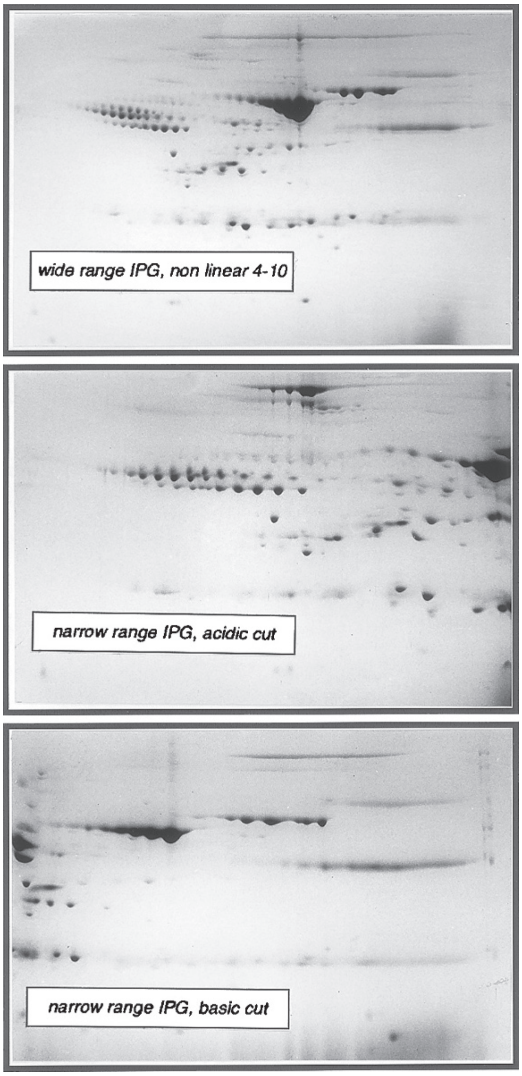

wide range IPG, non linear 4-10
narrow range IPG, acidic cut
narrow range IPG, basic cut

Fig. 3. Examples of IPG-DALT separations. Rat serum was run in 1-D on the 4–10 nonlinear IPG in **Table 2** (I) (top panel) (*27*) and on its narrow cuts, also in **Table 2** (acidic region (II) in middle panel, basic region (IV) in bottom panel). The 2d run was on a 7.5–17.5% T PAA gradient with the discontinuous buffer system of Laemmli (*28*); the samples from narrow range IPGs were overrun in order to improve resolution along the *Y* axis.

and basic protein components **(Fig. 3)** while maintaining the same slope ratios among the different pH regions. Alternatively, resolution may be improved by the parallel use of two linear, partly overlapping gradients, namely 4–7 and 6–10 ***(12)*** (**Table 3**).

Table 3
IPG 4–7 and 6–10, with a linear course[a]

Recipes for 5.1 + 5.1 mL gradients

	pH 4–7		pH 6–10	
chemicals	A[b] µL	B[c] µL	A µL	B µL
pK 3.6	197	103	329	35
pK 4.6	37	251	–	–
pK 6.2	153	51	96	117
pK 7.0	–	91	85	127
pK 8.5	–	–	91	83
pK 9.3	–	298	99	114
1M acetic acid	–	–	–	40
T30 C4	700	700	700	700
87% glycerol	1000	–	1000	–
TEMED	3.1	2.7	2.8	2.7
40% APS[d]	4.75	4.75	4.75	4.75
pH at 25°C[e]	4.02	6.93	5.99	9.72

[a] from ***(12)***
[b] acidic solution
[c] basic solution
[d] to be added to the solutions after transferring to the gradient mixer and starting the stirrer
[e] pH of the limiting solutions

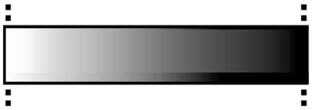
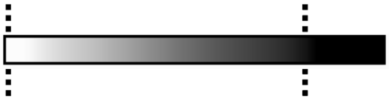
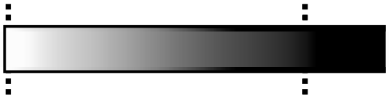
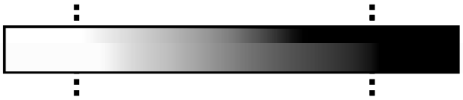
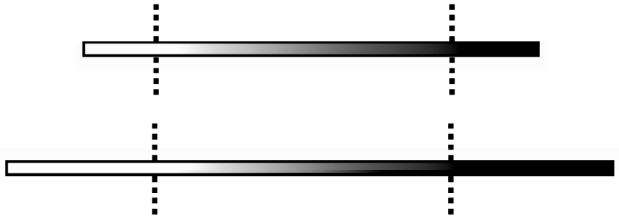
3.2. Selecting Size and Shape of the Gel Strips

In all 2-D protocols involving IPGs samples are run on individual strips. Depending on the available equipment/strip handling and on the requirements of the sample, to be shortly discussed below, alternative procedures may be applied (summarized in **Table 4**).

3.2.1. Strip Handling

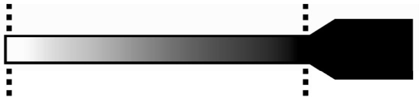
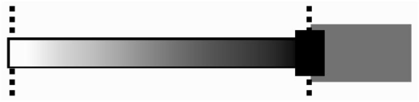
IPG strips are either run upside-up with sample applied ‘on-gel’, or the sample is rehydrated ‘in-gel’ (as part of the soaking solution) and the reswollen strips are run upside-down. The latter procedure is standard with ready-made commercial products; strips with comparable performance may be home-made along the protocols detailed in this chapter.

Table 4
Options in IPG strip geometry[a]

Custom geometry of the strips	Problem to be solved
A - optimal sample loading near either electrode, 'on gel application'	
1) 160[a] OR 80[c] x (between 10 and 20) mm^2, no plateau 	medium-high total protein load, higher sample volume; no spot distortion across sample loading area
2) (160+20) OR (80+20) x 7 mm^2, with plateau at either electrode 	proteins focusing at, or near to, optimal loading area
3) (160+20) OR (80+20) x 12 mm^2, with plateau at either electrode 	higher sample volume
4) (20+160+20) OR (20+80+20) x 12 mm^2, with plateau at both electrodes 	higher ionic concentration
B - optimal sample loading throughout the strip, 'in gel rehydration'	
(20+80+20) OR (40+80+40) x 3.5 mm^2, with plateau at both electrodes 	higher sample volume higher ionic concentration

(continued)

Table 4
(continued)

Custom geometry of the strips	Problem to be solved
C – special-purpose protocols	
1) Tall, wedge-shaped plateau 	*extremely* high sample volume (typically, as a result of problems / mistakes in dilution)
2) High-M_r ligands in plateau[d] 	*in situ* affinity subtraction

[a] from *(23)*

[b] reference is made to medium size slabs for second dimension, 160 mm wide; similar concepts apply when rescaling the protocols to a different standard size

[c] two 80 mm-long strips can be mounted per second dimension slab, head-to-tail, after cutting the plateau area; this arrangement is valuable with low total protein load, or running a high number of routine samples

[d] from *(30)*

3.2.2. Strip Shaping

Either the IPG slab is reswollen as a whole and grooves are then cut to leave parallel lanes on a continuous plastic backing (GelBond™), or the dry IPG is sliced together with its support and the strips independently reswollen.

The characteristics of the sample relevant for the choice of the loading procedure hence of the geometrical features of the IPG strips are (a) stability vs. pH, (b) volume and (c) salt content.

1. With some samples, only anodic or cathodic application performs well and avoid horizontal streaking of some proteins; this behavior disfavors in-gel rehydration.
2. When the strips are run upside-up, it is easy to cut strips several millimeters in width to arrange for higher protein loads and/or just larger sample volumes ***(21)*** (case A1 in **Table 4**). Sometimes however the optimal loading area (anodic, cathodic) overlaps the pI of relevant sample components, blurring/distorting their focusing pattern. Polymerizing an extension to the IPG, with constant pH, slightly higher at the cathode, slightly lower at the anode than the gradient bounds – polymerizing an immobilized pH (IP) plateau ***(21)*** – allows room for sample application without interference (A2). Increasing in addition the

width of the whole strip (A3), or wedge-shaping the plateau (C1) ***(22)***, lets higher and higher sample volumes to be loaded. With thin IPG strips to be run upside-down it is possible to use IP plateaus to increase the protein load. As an example, next to a pH gradient 8 cm in length anodic and cathodic plateaus may be cast to match electrode-to-electrode distances of 10.5 or 16 cm (B), typical of standard focusing trays, approximately doubling sample volume ***(23)***.

3. When it is known, or feared, that the samples contain higher amounts of salts, and dialysis is impractical, pH plateaus at both electrodes (A4) may collect the ionic ridges past the anodic or cathodic application point and allow for complete migration of the sample proteins ***(24)***. Using this procedure, up to 12.5 µmoles of KCl are compatible with an upside-down strip with 4 + 4 cm IP ***(23)***. With upside-up strips, changing the electrodic paper strips after some hours of run further helps easing the extremes of pH and conductivity beside the electrodes **(22, 25)**.

The recipe for a non-linear 4–10 IPG with IP plateaus in given in **Table 5.**

Table 5
IPG 4-10, with a non-linear corse, with electrodic IPs[a]

	Recipes for 2.7 mL solutions[b]			
chemicals	AIP[c] µL	A[d] µL	B[e] µL	BIP[ef] µL
pK 0.8	78	73	8.5	6
pK 4.6	69	69	-	-
pK 6.2	81	81	24.5	24.5
pK 7.0	-	-	32.5	32.5
pK 8.5	-	-	12	12
pK 9.3	-	-	23	23
1M acetic acid	-	-	7.5	7.5
T30 C4	360	360	360	360
87% glycerol	830	680	150	-
TEMED	1.65	1.65	1.43	1.43
40% APS	2.5[g]	2.5[h]	2.5[h]	2.5[g]

[a] from ***(14)*** and ***(23)***

[b] to prepare a 16 cm-high gel matrix in a 12.5 *x* 25 cm, w *x* h, polymerization cassette

[c] solution for acidic IP

[d] acidic solution

[e] basic solution

[f] solution for basic IP

[g] to be added to the solutions in the test tube, before transferring to the polymerization cassette with a pipet

[h] to be added to the solutions after transferring to the gradient mixer and starting the stirrer

3.3. Preparing the Working Solutions

As an equilibrium technique, IPG technology freezes within the structure of the amphoteric matrix the actual composition of the acrylamido buffer mix. Since there is no adjustment once the gradients are cast, all steps in liquid handling require the careful use of properly calibrated measuring devices.

1. On two calibrated test tubes or 10-mL cylinders, mark the pH and the volume of the solutions to be prepared with a felt-tip (*see* **Note 1**).
2. Add the acrylamide buffers. For reproducible measurements, all chemicals should be at room temperature (*see* **Note 2**).
3. After adding all the acrylamido buffers, fill the two solutions to one-half their final volume with water, and check their pH with a microelectrode. Refer to expected pH readings at 25°C in the liquid phase, not the values computed for 10°C in the gel phase (*see* **Tables 2** and **3**).
4. Add acetic acid to the basic solution to bring the pH to between 7.0 and 7.5 in order to prevent hydrolysis of the amide bonds during the polymerization step.
5. Add glycerol to the acidic solution to a final concentration of 15–20% v/v. Use a widemouth or a clipped pipet tip for dispensing the 87% glycerol stock.
6. The solutions are filled to their final volume with water and carefully mixed. Acrylamide is typically used at the final concentration of 4% T, 4% C. The concentration of TEMED is higher in the acidic than in the basic solution to counteract the effect of amine protonation. Ammonium persulfate is **not** added at this stage (*see* **Note 3**).

3.4. Assembling the Polymerization Cassette

To obtain a 16-cm high IPG slab that fits the 2-D SDS-PAGE gel (e.g., Protean™ from Bio-Rad [Hercules, CA]), the 12.5 *x* 25 cm w *x* h cassette is used (*see* **Note 4**).

1. When preparing slabs to be reswollen as a whole, mark a GelBond PAG™ foil for polarity using a felt-tip marker (anode at the bottom, cathode at 2/3 height); additional annotations (pH gradient, date, even short reminders) may help further identification. The felt-tip marking does not interfere with electrophoretic procedures (*see* **Note 5**). When preparing strips to be individually reswollen, a "code" for identifying gradient type and polarity may be devised, using felt-tip pens of different colors and a variable pattern of parallel lines.
2. Wet the gel-supporting plate with distilled water.
3. Lay the GelBond PAG™ foil—hydrophobic side down, hydrophilic side up, still covered with the protective paper foil—on a gel-supporting plate. Adhere the foil to the plate by rolling it onto the glass. Take care to align the foil flush with the plate.
4. Discard the protecting sheet. Blot any water on the hydrophilic side of the foil with a paper tissue.
5. Swab the inside of the molding plate with a paper towel moist with a dimethyldichlorosilane solution. It is recommended to wear gloves and to perform this treatment in a fume hood.

6. After the solvent has dried out, rinse the surface of the glass with distilled water, and dry with a paper towel. Inspect the rubber gasket for any break or any adherent material (driedout acrylamide, paper cuts, and so forth).
7. Invert the molding plate on the GelBond-glass assembly, and carefully align it. Check the marked polarity!
8. Fasten the cassette with clamps of adequate thickness and strength. If two strips from the 1-D IEF are to be aligned tail-to head for the 2-d SDS-PAGE run on the same gel, the procedure detailed above applies to the 25 *x* 12.5-cm w *x* h mold. Pouring a gradient of the stated volume **(Table 2)** will result in an 8-cm-high gel.

3.5. Pouring the Gradient

3.5.1. Pouring the Gradient without IPs

1. Add the magnetic bar to each chamber of the gradient mixer. Set both connections between the two vessels as well as to the outlet tubing in the closed position. Arrange the mixer on a stirrer, about 5 cm above the mouth of the polymerization cassette.
2. Stick the outlet(s) (*see* **Subheading 2.1., item 2**) in the cassette taking extreme care to not miss the opening to reach instead the gap between GelBond and supporting glass plate.
3. Transfer the acidic, heavy solution into the mixing chamber of the gradient mixer (i.e., the vessel next to the outlet).
4. Briefly open the connection to the reservoir in order to purge air bubbles from the solution; return the solution that flowed into the reservoir during the purging operation to the mixing chamber with a Pasteur pipet.
5. Add the basic light solution to the reservoir.
6. Start the mixing on the stirrer at high speed. Slowly move the gradient mixer around the active area of the mixer in order to find a position in which the magnetic bars in both chambers turn evenly and synchronously. Reduce the stirring speed.
7. Add ammonium persulfate solution in the stated amounts (*see* **Table 2**) to the solutions in both chambers.
8. Open the outlet to the polymerization chamber; the liquid should easily flow by gravity: in case it does not, a light pressure (the palm of your hand over the mouth of the vessel) might solve the problem.
9. When the heavy solution is about to enter the polymerization cassette, open the connection between the two chambers of the mixer.
10. The in-flowing light solution should mix promptly with its heavy counterpart; if not (streams of liquid of different density are distinctly seen in the mixing chamber), the stirring speed should be increased accordingly (*see* **Note 6**).
11. When all solution has flown by gravity, force the few drops remaining in the outlet tubing and in the mixing chamber to the cassette by applying pressure *(see above)* first on the reservoir and then on the mixing chamber, while raising the gradient mixer.
12. Inspect the liquid within the cassette: no air bubbles should be present. It is possible to remove them, with **great caution**, with the help of a thin hook cut from GelBond foil.

13. Leave the gradient to stabilize for 5–10 min at room temperature before moving the cassette into the oven to start the polymerization step.
14. After use, promptly fill the gradient mixer with distilled water and flow the water through the outlet tubing; repeat this procedure twice.
15. Set aside the stirring bars; disassemble the outlet tubing and dry by suction with a pump; and blow the liquid still remaining inside the gradient mixer with compressed air, before drying the outside with a paper tissue.
16. Let the equipment dry to completion with all connections in the open position. Rinsing and drying the gradient mixer, and pouring a new gradient takes 10 min, so no delay is actually required when processing a number of IPGs one after another.

3.5.2. Pouring the Gradient with IPs

1.-6. Same as in **Subheading 3.5.1.**

7.1. Add ammonium persulfate solution in the stated amounts (*see* **Table 5**) to the test tube containing the solution for the acidic IP; mix (inversion of the tube topped by Parafilm, or vortexing); transfer to the polymerization cassette with a Pasteur pipet.

7.2. Add ammonium persulfate solution in the stated amounts (*see* **Table 2**) to the gradient solutions in both mixer chambers.

8.-11. Same as in **Subheading 3.5.1.**

12. Add ammonium persulfate solution in the stated amounts (*see* **Table 5**) to the test tube containing the solution for the basic IP; transfer to the polymerization cassette with a Pasteur pipet.

13.-16. Same as in **Subheading 3.5.1.**

3.6. Polymerization Conditions

1. Let the IPGs polymerize for 1 h at 50°C (*see* **Note 7**).
2. Gel setting occurs within approx 10 min, whereas polymerization slowly proceeds to plateau in the next 50 min. It is critical during the early period that the polymerization cassette is kept in an upright position **on a level surface**. Moreover, the oven should not be opened in order to avoid both shaking the cassette and causing the temperature to drop.

3.7. Washing, Drying, and Storing IPGs

1. At the end of the polymerization period, disassemble the cassette by removing the clamps.
2. Insert a scalpel blade between GelBond and glass plate to loosen the foil from its support.
3. Fill a plastic box in which the gel may lay flat with 1 L of glycerol washing solution.
4. Gently peel the IPG from the mold, and transfer (upside up!) into the box containing the glycerol solution.
5. Gently shake for 1 h at room temperature.

6. Lay the gel (upside up!) on a supporting glass plate partially or completely submerged in the washing solution in order to avoid trapping air bubbles (*see* **Note 8**).
7. Set the assembly upright, approx 30 cm in front of a (cool) fan, operating at high speed. The whole area should be as dustless as possible; glycerol smudge is better collected on a tray.
8. Evaluate the progression of the drying by sensing the cooling from water evaporation on the back of the glass plate and by looking for an even and matt appearance on the front of the gel. Depending on the temperature and relative humidity, the drying step may take between 1 and 2 h, and should not be unnecessarily prolonged. It is acceptable, but not recommended to let the gels dry unattended (e.g., overnight, at room temperature).
9. For barely damp gels, the very last phase of drying may occur in the refrigerator, with the gel in an open box. If needed, the polymerized gels may be stored overnight in their cassettes at 4°C before washing.
10. The dry gels may be further processed right away or stored for later use. Dry IPGs may be kept in boxes at 4°C for few days. For longer periods of time ***(26)***, they are stored at −20°C. For use along the in-gel rehydration protocol, the dry IPG matrix is carefully covered with Parafilm, minimizing trapped air bubbles. The assembly is then sliced with a paper cutter to obtain 3.5 mm-wide strips, to be stored in ziplock bags at −20°C.

4. Notes

1. All required stock solutions are orderly aligned on the bench. It is most helpful if their sequence corresponds to the order in which they are called for by the recipe. This is especially important for the acrylamido buffers, whose bottles look necessarily alike, whereas the pK label is in a relatively small print. It may be advisable to duplicate the pK label on the bottle caps, in larger print, with felt-tip pens. It is strongly suggested that the recipe be marked with a ruler and that each chemical be put back in a box after its use. When a bottle is open, hold its cap upside down on a paper tissue. Make sure you are storing the open bottles on a flat, stable surface (like a tray): Expensive and not harmless Immobiline may be easily if inadvertently spilled.
2. Positive displacement measuring devices might be more accurate than air-displacement pipets. No liquid droplets must adhere to the outside of the pipet tips; for this purpose, it is usually sufficient to touch the neck of the bottle. For a quantitative transfer of the Immobiline chemicals, and especially of the basic monomers dissolved in an alcoholic solution, two precautions should be taken; namely, slow pipeting and rinsing the pipet tips twice with distilled water after each measurement.

3. It is not customary to prepare large batches of the limiting solutions—the storage conditions in the mixture are appropriate for none of the chemicals. However, leftover solutions might be used within a couple of days.
4. In the case where the commercial products were not available, an adequate substitution for the gel mold may be a plate of plain glass (polished and saturated with silane) together with a rubber gasket of approx 0.5-mm thickness. The latter may be cut from foils of *para*-, silicone, or nitrile rubber, should have a U-shape, and should be ~5 mm wide. Thick clamps from stationery stores may then be used, but care should be taken to apply the pressure on the gasket, not inside it.
5. A note of caution for 1-D experiments with radioactive samples. In some cases, the ink gives a strong positive signal; more commonly, the writing negatively interferes with film exposure. In these experiments, all marks should then be outside the area of sample application (where their disturbing appearance may turn into the definition of useful reference points).
6. The major points of caution will be stressed once more below. The flow between the different compartments must be unhindered by air bubbles between the two vessels of the mixer or by water droplets in the outlet. No backflow should occur. The pouring should not be too fast to allow proper mixing in the chamber and to avoid turbulence in the cassette (with a proper selection of the pressure drop). The solution should evenly flow along the hydrophilic wall of the cassette (lined with GelBond) and not fall dropwise along the hydrophobic one.
7. A forced ventilation is more appropriate to this purpose than a convection oven in order to provide even and controlled heating. It should be recalled that the selected temperature is the one allowing identical incorporation efficiency for all acrylamido buffers, which grafts into the gel matrix a pH gradient exactly matching the computed course ***(23)***.
8. It is sensible to set aside some containers only for this purpose, i.e., to avoid recycling between gel washing, protein staining, and blot immunodetection. Owing to its high viscosity, 100% glycerol should be avoided as stock reagent in favor of the 87% preparation; ~12 mL of the latter may be measured in a small plastic beaker instead of in a graduated cylinder. Failure to include glycerol as a humidity-conditioning agent results in slab curling and easy peeling and tearing of the gel from its support. On washing, IPGs reswell to a different extent, depending on the pH of the matrix and on the course of the glycerol gradient.

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A Modified Nonequilibrium pH Gradient Electrophoresis (NEPHGE) Technique for Protein Separation

James A. Carroll

1. Introduction

Membrane proteins, hydrophobic proteins, and proteins with alkaline isoelectric points (pIs) traditionally have been difficult to visualize by two-dimensional (2-D) gel electrophoresis. There are a multitude of reason as to why proteins with basic pIs (many of them membrane proteins) are difficult to resolve by standard IEF methods: (i) the lack of pH gradient formation above pH 7.6 due to the buffering effect of urea, (ii) precipitation of proteins when entering the gel and during focusing, (iii) cathodic drift during focusing resulting in the migration of basic proteins off the ends of the tube gels, or (iv) lack of solubility in particular nonionic detergents. O'Farrell et al. first reported in 1977 a novel protein separation technique termed Nonequilibrium pH Gradient Electrophoresis (NEPHGE) as a method to enhance the separation of proteins with significantly basic pIs ***(1),*** but this alternative technique had its limitations due to issues with reproducibility with the reagents of that era. With the advent of new compounds for the solubilization of recalcitrant proteins, my laboratory has made minor modifications to the original NEPHGE protocol reported by O'Farrell offering enhanced reproducibility with increased solubilization of alkaline and membrane proteins ***(2–4).***

When performing NEPHGE, the pH gradient does not reach steady-state (as is the case with IEF) and shorter focusing times are applied. Acidic proteins will focus to completion but the basic proteins do not, rather they are continually migrating in the electrical field towards their pI. The electrophoretic pattern will change depending on the accumulation of volt hours (Vhr) applied during focusing; therefore, it is necessary to empirically determine the optimal Vhr yielding the

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desired resolution. Furthermore, it is important to note that the polarity of the focusing tank must be reversed in order to perform NEPHGE.

2. Materials

2.1. NEPHGE Reagents

1. C_4TT (CHAPS/Triton/Thiourea) NEPHGE buffer for solubilization of proteins: 7 *M* urea, 2 *M* thiourea, 4.0% CHAPS (w/v), 1.0% Triton X-100 (v/v), 65 m*M* DTT, and 2.0% preblended ampholytes pH 3 to 9.5 (Amersham, Piscataway, NJ) (v/v). Prepare 10 to 20 ml, sterile filter (0.45 µm), and store at −80°C until use. Shelf life is approximately 1 year.
2. NEPHGE overlay solution: C_4TT NEPHGE buffer mixed 1:1 with dH_2O. Store at −80°C until use. Shelf life is approximately 1 year.
3. NEPHGE tube gel solution: 8 *M* urea, 4.4% Duracryl acrylamide (30 T, 2.6C) (Genomic Solutions, Ann Arbor, MI), 4.0% CHAPS (w/v), 1.0 % Triton X-100 (v/v), and 2.0% preblended ampholytes pH 3 to 9.5 (Amersham, Piscataway, NJ) (v/v). This solution should be prepared fresh each time tube gels are to be poured.
4. SDS equilibration buffer: 0.125 *M* Tris-base (pH 8.8), 3 *M* urea, 2.0% SDS (w/v), 10% glycerol (v/v) in dH_2O. Prepare 200 to 500 ml, sterile filter, and store at room temperature. Measure the necessary volume for several equilibrations and add DTT to final of 100 m*M* before application.
5. Ammonium persulfate (APS) stock solution: 10% APS in dH_2O. Store at 4°C protected from light. Shelf life is 2 to 3 weeks.
6. Bromophenol blue stock solution: 0.1% (w/v) in dH_2O stored at room temperature.
7. Anode solution*:* 6.0 m*M* H_3PO_4 in dH_2O made fresh (1 liter). Degas under vacuum for 15 minutes
8. Cathode solution: 20 m*M* NaOH in dH_2O made fresh (2 to 3 liters). Degas under vacuum for 15 minutes.
9. Agarose sealing solution*:* 1.0 % agarose in 50 m*M* Tris-base, 0.15% SDS (w/v), 384 m*M* glycine in dH_2O. Add 500 µl of bromophenol blue stock solution to 250 mLagarose sealing solution. Store at room temperature.

2.2. SDS-PAGE Reagents

Standard SDS-PAGE in the second dimension at a variety of final acrylamide percentages. We typically separate our protein mixtures using a final concentration of 12.5 % acrylamide.

3. Methods

3.1. NEPHGE Sample Preparation

For sample preparation, proteins can be precipitated from cell lysates or protein fractions by the addition of 1 volume saturated TCA and 8 volumes of ice cold acetone. The sample is incubated for 1 hour at −20°C and proteins recovered by centrifugation (16,000 × *g*, 4°C, 20 min). The protein pellet is washed in 80% ice cold acetone in dH_2O, centrifuged again, and briefly air dried to remove residual acetone. Alternatively, rinsed pellets of cells or tissue can be used without precipitation. Proteins are suspended in C_4TT NEPHGE buffer to a concentration of 5 to 10 µg/µl and incubated overnight (approximately 16 hours) at 23°C with gentle agitation. Samples are subsequently clarified by ultracentrifugation (≥ 200,000 × *g*, 30 min, 23°C) to remove insoluble material that may inhibit focusing (*see* **Note 1**). Samples can be loaded immediately onto NEPHGE tube gels or stored at −80°C until use.

3.2. Casting NEPHGE Tubes and Slab Gels

1. Similar to the IEF protocol, draw a line on the clean glass tubes at the desired height (12 to 16 cm depending on the slab gel apparatus used in the second dimension). We typically use glass tubes with an internal diameter of 1.0 or 2.0 mm. A stack of tubes (approximately 20 to 30) is placed vertically into a clean glass 100 mLgraduated cylinder for casting.
2. To cast the NEPHGE tube gels, mix 7.21 g urea, 2.0 mL Duracryl acrylamide, and 0.3 gm CHAPS in 6.5 mL dH_2O until the urea is dissolved (caution: do not heat this mixture).
3. Add 750 µl preblended ampholytes pH 3 to 9.5 (Amersham, Piscataway, NJ) to the mixture and bring up to 15 mLwith dH_2O. Invert several times to mix without the introduction of bubbles and degas under vacuum for 5 minutes.
4. Add 70 µL of 10 % APS and 10 µl TEMED and mix again by gentle inversion of the tube.
5. Immediately pipet the entire amount into the graduated cylinder holding the glass tubes for casting **(Figure 1).**

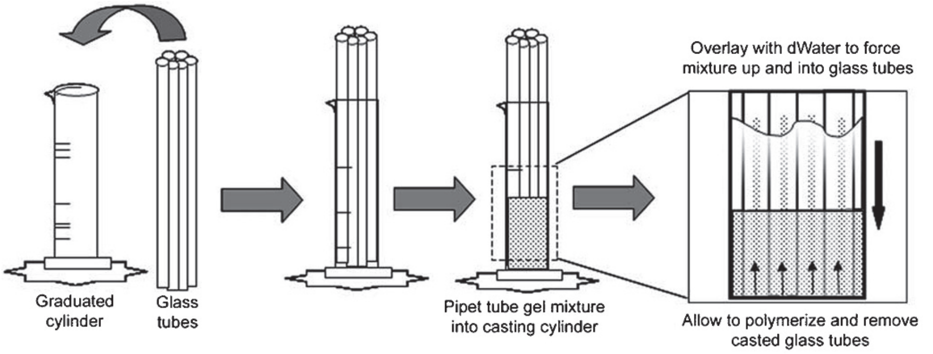
Graduated cylinder
Glass tubes
Pipet tube gel mixture into casting cylinder
Overlay with dWater to force mixture up and into glass tubes
Allow to polymerize and remove casted glass tubes

Fig. 1. Pouring NEPHGE tube gel**s.** Place the bundle of clean glass tubes into a clean graduated cylinder. Slowly pipet the NEPHGE acrylamide mixture into the casting cylinder, while avoiding bubbles. Gently overlay dH_2O using a squirt bottle, forming an emulsion layer, and force the NEPHGE acrylamide mixture into an up the glass tubes. Apply dH_2O until the level of the NEPHGE acrylamide mixture reaches the desired height inside the glass tubes.

6. Immediately use a squirt bottle to gently apply dH_2O, forming an emulsion layer of water atop the NEPHGE gel mixture. This forces the NEPHGE gel mixture up into the glass tubes by hydrostatic pressure. Apply water until the gel mixture reaches the marked level on your glass tubes.
7. Allow to polymerize for at least 2 hours at room temperature. Once polymerization is complete, pour off the water overlay and gently twist each glass tube gel and remove from the graduated cylinder casting stand. Look for air bubbles or malformations in the tube gels. Poured tube gels can be stored in a plastic bag for up to 2 weeks at 4°C, but best results are obtained when focused immediately.

3.3. Focusing NEPHGE Tube Gels

1. Load the protein sample solubilized in C_4TT NEPHGE buffer (up to 200 µg of protein) onto the tube gel using a 50 or 100 µLHamilton syringe. We typically dilute the sample so that we load approximately 50 to 100 µL of sample per tube gel.
2. Gently overlay the protein sample with 50 µl NEPHGE overlay solution using a Hamilton syringe to protect the sample from acid hydrolysis. A line of demarcation should be visible between the sample and the overlay as an emulsion layer forms.
3. Fill the remainder of the glass tube headspace with 6 m*M* H_3PO_4 (anode solution) using a long pasture pipet.
4. Fill the lower chamber with the cathode solution (20 m*M* NaOH).
5. Insert the NEPHGE glass tubes into the electrophoresis chamber and place into the lower chamber.
6. Add the anode solution until the tops of the glass tubes are submerged.
7. Connect the electrophoresis chamber to the power supply, but remember to *REVERSE* the polarity of the unit (*see* **Note 2**). We simply reverse the leads.
8. Electrophoretic focusing begins at 200 V for 1 hour and is then increased to 600 V for 4 hours (a total of 2600 Vhr) (*see* **Notes 3** and **4**). Times will vary dependent on the degree of separation desired and must therefore be empirically determined **(Fig. 2).**
9. With focusing complete, tube gels are extruded by force using a 3 mLsyringe filled with dH_2O with a piece of flexible tubing to attach it to the glass tube and stored at −80°C until separated in the second dimension by SDS-PAGE. Best results are achieved when focused NEPHGE tube gels are immediately separated by SDS-PAGE in the second dimension.

3.4. Applying NEPHGE tube gels onto the second dimension

1. To separate the proteins by SDS-PAGE, the tube gels must be equilibrated in SDS equilibration buffer with fresh 100 m*M* DTT.
2. Add 10 mLof SDS equilibration buffer with DTT and rock for 10 minutes at room temperature, and repeat.
3. Apply the tube gel to the top of a 1.5 mm SDS-PAGE gel. We typically lay the tube gel onto a 13.5 cm × 1.5 mm × 14 cm (L × W × H) 12.5% acrylamide gel using a Hoefer

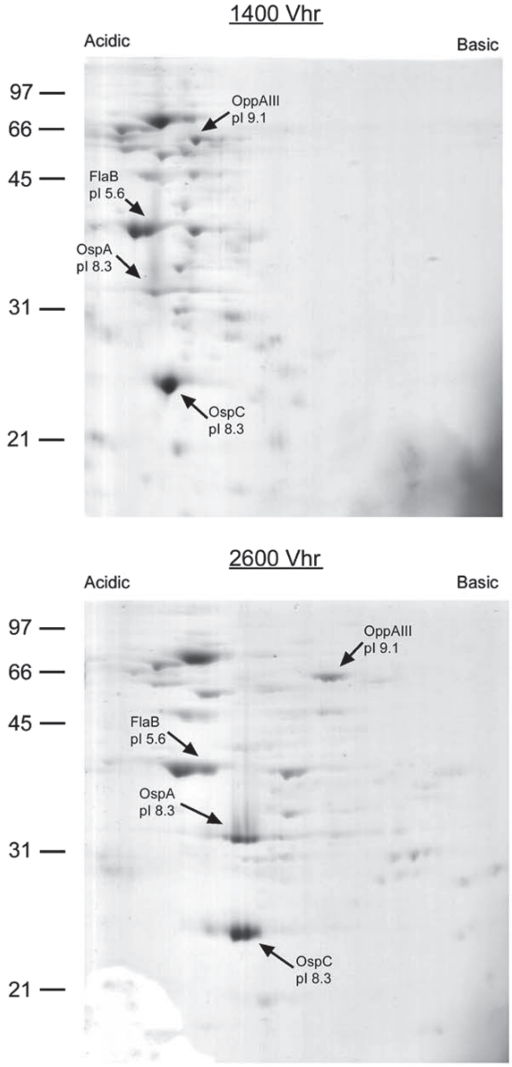
1400 Vhr
Acidic
Basic
97
66
45
31
21
OppAIII
pI 9.1
FlaB
pI 5.6
OspA
pI 8.3
OspC
pI 8.3
2600 Vhr
Acidic
Basic
97
66
45
31
21
OppAIII
pI 9.1
FlaB
pI 5.6
OspA
pI 8.3
OspC
pI 8.3

Fig. 2. Separation of Membrane Proteins from *Borrelia burgdorferi* by NEPHGE. Membrane-associate proteins from *B. burgdorferi* were separated in the first dimension by NEPHGE on 15 cm tube gels for a total of 1,400 or 2,600 Vhr. After focusing was halted, the tube gel was equilibrated with SDS, separated in the second dimension by 12.5% SDS-PAGE, and stained with Coomassie blue. Acidic and Basic ends of the gel are indicated. The positions of several proteins (OppAIII, FlaB, OspA, and OspC) are labeled and are accompanied by their theoretical pI. Relative molecular mass standards are indicated to the left of each gel. Note how the resolution of proteins differs with increasing Vhr.

SE600 gel apparatus. Be careful that no bubbles are introduced between the tube gel and the polyacrylamide gel.

4. Once the gel is in place, gently place the upper SDS-PAGE chamber on top of the tube gel/polyacrylamide gel. Seal in the tube gel with approximately 5 mLof warm agarose sealing solution so that the tube gel will not float out of position during filling of the chamber with buffer or during electrophoresis.
5. Perform SDS-PAGE to resolve proteins as described.

4. Notes

1. It is important to reduce the amount of nucleic acid, salt, polysaccharide, and lipid contamination in the sample material for optimal separation. These abundant molecules can inhibit separation by physical obstruction of the acrylamide matrix or by altering the conductivity of the sample. This can be accomplished in part by precipitation prior to solubilization and clarification of the sample by ultracentrifugation prior to loading.
2. NEPHGE requires that the anode buffer and cathode buffer be reversed compared to standard IEF. Remember to reverse the polarity of the electrophoresis apparatus.
3. Due to the nature of the procedure where the pH gradient does not reach equilibrium, solubilized proteins are typically migrating towards their pI. Close attention to the accumulated Vhr **(Fig. 2)** is required for reproducibility and must be empirically determined by the investigator.
4. Occasionally, proteins will precipitate as they move from the liquid phase into the semi-solid phase of the tube gel, resulting in sample loss. This precipitation event can be reduced by the inclusion of a small amount of Sephadex G-200 between the sample and tube gel ***(5).***

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Microchip Capillary Electrophoresis

Application to Peptide Analysis

Barbara A. Fogarty, Nathan A. Lacher, and Susan M. Lunte

1. Introduction

Peptides are a large class of molecules that differ in size, charge, conformation, hydrophobicity, and the ability to form biospecific complexes. Peptides play an important role in many physiological processes, including the regulation of pain, blood pressure, and immune response, and can act as antibiotics, coenzymes/enzyme inhibitors, drugs, growth stimulators, hormones, neurotransmitters, and toxins ***(1).***

Since the identification of leu-enkephalin in the 1970s, the role of neuropeptides in the regulation of the central nervous system and signal transduction has been extensively investigated ***(1).*** Identification of specific peptides and their functions may permit the design of pharmacologically active synthetic analogs that could be used for the treatment of neurological diseases such as Parkinson's and Alzheimer's. Peptide analysis is also important for proteomics research. Digestion of a protein yields distinct peptide mixtures, which are separated and mapped in order to characterize the parent molecule. In some cases, multidimensional separations are needed to achieve resolution and identification of all peptide components.

1.1. Considerations for Peptide Analysis

The determination of peptide concentrations in complex biological samples, protein digests, and drug formulations demands the use of highly efficient, sensitive, and selective analytical methods. Electrophoretic separation techniques are routinely employed due to their fast analysis times, high separation efficiencies, and compatibility with sensitive detection techniques ***(2).*** Electrophoretic-based separation modes have been used for the determination of peptide mobility,

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isoelectric point, relative mass and charge, dissociation/association constants of complexes, diffusion coefficients, and hydrophobicity *(3).*

For enzyme and immunoassays where biological activity must be maintained, buffers of physiological pH and temperature can be used. For hydrophobic peptides, the addition of surfactants to the run buffer can aid peptide solubility. Coating the capillary wall is also used to minimize adsorption of analyte to the capillary surface *(4)*. Buffer conditions must also be optimized if mass spectrometry is used for detection due to the possibility of ion suppression. Lastly, since most peptides are amphoteric, analyte charge can be altered by adjusting the pH. This can change mobility, as well as enhance solubility and reduce adsorption to the negative wall.

Many of the methods developed for peptide analysis on conventional capillary electrophoresis systems are now being transferred to microchip platforms due to the many additional advantages of miniaturized analytical systems *(5).* This chapter will discuss general considerations for peptide analysis using microchip CE, highlight different techniques that can be used, and look at some specific applications of this technology.

1.2. Microchip Devices

In microchip electrophoresis, separation is typically achieved through a combination of electrophoresis (μ_e) and electroosmotic flow (μ_{EOF}). Charged analytes migrate under the influence of an electric field and are separated due to

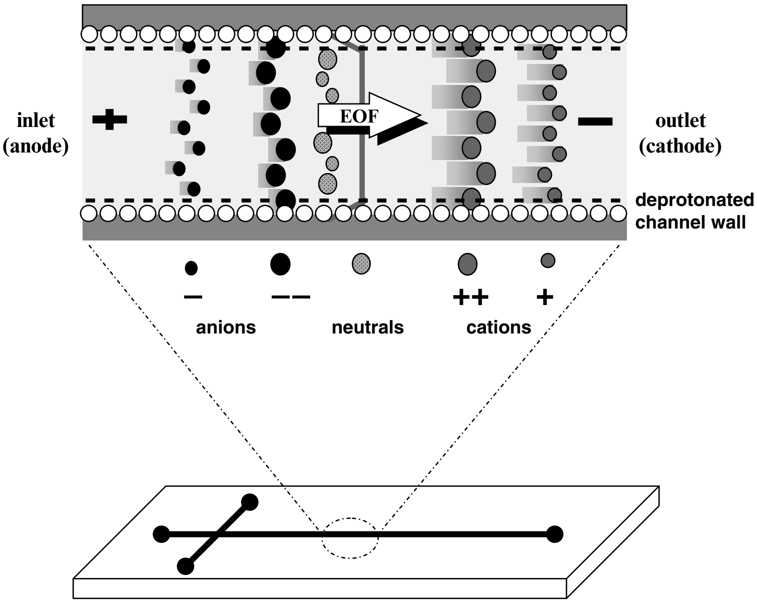

Fig. 1. Microchip capillary electrophoresis.

differences in their mass/charge ratios **(Fig. 1)**. Generation of EOF (a bulk flow that causes all ions, regardless of charge, to migrate to the cathode) is achieved through the ionization of silanol groups on the wall of the separation channel. The net negative charge on the channel wall attracts positively charged solvated ions from the buffer solution, forming an electric double layer. Upon generation of the electric field, the migration of solvated cations to the cathode creates a bulk flow of solution within the channel toward the cathode where the detection region is located. As long as the magnitude of the EOF is greater than the electrophoretic mobility of the analytes ($\mu_{EOF}>\mu_e$), all species in solution. regardless of charge will eventually pass the detector.

Separations on microchips are faster and more efficient, and higher sample throughput can be realized than with conventional systems. The ability to move small volumes of fluids around the chips makes it possible to analyze samples of limited volumes as well as integrate sample handling and preparation steps onto a single platform. The planar nature of microchip devices also facilitates the use of 2-D separation mechanisms for the resolution of complex mixtures. This will be discussed in further detail later.

Microchip devices have been fabricated in a number of different substrates. Glass devices fabricated in soda lime, borosilicate, and quartz substrates have surface chemistries similar to that of the fused silica capillaries used in conventional systems. This allows the direct transfer of conventional methods to microchip systems. However, glass microchips have limitations, including the time required for fabrication, high cost, and fragile nature of the devices. In addition, integration of functional elements, such as separation and detection electrodes, is challenging due to the high temperatures needed for the glass bonding process.

Inexpensive polymer substrates, including poly(methylmethacrylate) (PMMA) and poly(dimethylsiloxane) (PDMS), have been employed for the production of low-cost microchips ***(6).*** These disposable devices eliminate issues of sample carryover and cross-contamination. However adsorption of peptides to hydrophobic polymers such as PDMS can be an issue ***(7).*** In addition, PDMS generates a reduced EOF relative to that of glass, which can influence the efficiency of separation (*see* **Note 1**).

Approaches to minimize peptide adsorption to channel walls include the use of dynamic and permanent modifications. Dynamic coatings have been used to minimize adsorption of hydrophobic peptides to channel surfaces. Polymers such as polyvinylpyrrolidine have been used to modify channels in a glass microchip, resulting in improved resolution of small test peptides ***(8).*** PDMS devices have also been modified with different monomers using UV grafting techniques ***(9).*** Reduced peak adsorption and improved peak efficiencies were observed for two model peptides, F-PKC and F-src. Poly(ethylene glycol) has also been grafted onto the surface of PMMA using atom-transfer radical polymerization ***(10).*** The modification resulted in a substantial reduction in EOF and nonspecific adsorption of proteins on the surface of the PMMA. The separation of a tryptic digest of BSA was demonstrated with the device. Dynamic coating of a PMMA microchip with sodium dodecyl sulphate (SDS) allowed the separation of proteins and peptides up to 116 kDa in size ***(11).*** Cathodic EOF was used as the driving force for the separation. For a more detailed look at approaches to the permanent surface modification of polymer-based electrophoretic devices, the reader is referred to a recent review of the area ***(12).***

The major advantages of using planar microchip devices for peptide analysis include the ability to integrate sample preparation, injection, separation, and detection steps onto a single platform. Approaches to each of these stages of peptide analysis with respect to microchip technology will be examined in further detail.

2. Methods

2.1. Sample Preparation

Preparation of peptide samples prior to analysis can involve dialysis, filtering, or extraction techniques to ensure that the sample is as clean as possible, preferably free from proteins and salts. Techniques such as solid-phase extraction can be used for preconcentration of peptides present at low concentrations in complex mixtures. While sample preparation is generally carried out offline, the integration of extraction, dialysis, and digestion steps for the development of a true miniaturized total analysis system has been implemented with some success. However, much of the work in this area has been proof-of-concept with limited application to real-world samples thus far ***(13).***

2.1.1. Sample Preconcentration and Desalting

Extraction and preconcentration of peptides for electrophoretic analysis is traditionally achieved using conventional chromatographic stationary phase materials. To use conventional stationary phase particles in microfluidic devices, a mechanism for effective particle containment is needed to prevent clogging of microchannels. This is typically achieved using tapered reaction chambers to promote keystone packing of particles or the use of weir-like structures. Packed C18 particles have been used to preconcentrate and to remove salt and urea from peptide mixtures ***(14).***

Limitations of particle-based systems include the generation of high back-pressures and the potential for clogging. One solution to these problems involves the use of polymer monolith materials. Advantages of these continuous bed systems for sample extraction and preconcentration include low back-pressures, tailored chemistries and easy control of pore size. For microfluidic applications, monoliths can be formed *in situ* and polymerized using heat or UV light. Methacrylate-based monolith systems have been employed for preconcentration of the hydrophobic tetrapeptide Phe-Gly-Phe-Gly by a factor of 1000 ***(15).*** Lauryl methacrylate monoliths have been polymerized in SU8 channels for desalting and subsequent separation of a cytochrome c digest peptide digest ***(16).*** Further optimization of column dimension was needed to improve the separation of the fragments. A PMMA substrate was modified with zeolite nanoparticles for the digestion and enrichment of protein samples ***(17).*** Trypsin immobilized in a sol-gel matrix was used to form a bioreactor for the digestion of cytochrome c and bovine serum albumin with a reaction time of under 5 s.

A 2-D approach to sample preparation and desalting has also been described ***(18).*** The chip device contains two channels with integrated conductivity detectors. A combination of isotachophoresis followed by capillary zone electrophoresis was used for the preconcentration of myoglobin and for the desalting of a protein mixture. The salt was removed during the ITP step, allowing subsequent separation of proteins in the second channel.

2.1.2. Microdialysis

Microdialysis is an *in vivo* sampling technique used for continuous monitoring applications. Probes are implanted in biological tissues and fluids to monitor biological events such as neurotransmitter release. A membrane with a predetermined molecular weight cutoff is used to exclude large molecules such as proteins while allowing smaller molecules such as amino acids and peptides to pass. This technique has recently been coupled to microchip electrophoresis ***(19).*** **Fig. 2** shows a microchip CE separation of two fluorescently labeled peptides following on-line recovery through a microdialysis probe ***(19).*** This system has been further improved by the incorporation of an online labeling protocol for the detection of a peptide mixture that included Leu-enkephalin **(19).**

2.1.3. Protein Digestion: Proteomic Applications

Proteolytic enzymes such as trypsin are used for the cleavage and digestion of protein molecules into their respective peptide fragments prior to identification/sequencing. Trypsin-coated beads have been employed for the on-chip digestion of proteins in microchip CE devices. High bead-to-peptide mass ratios and efficient mass transfer can permit rapid digestions of proteins including melitten, cytochrome c, bovine serum albumin, and β-casein. Digestion times ranged between 5 sec and 6 min at room temperature ***(20).*** This is in contrast to conventional protein digestion procedures that can often take up to 24 hours. Pressure or electroosmotic flow can be used to pump proteins through trypsin reactors ***(21).*** Localization of trypsin by enzyme adsorption to integrated membranes such as poly(vinylidene fluorine) has been used for the on-chip digestion of cytochrome c ***(22, 23).*** Trypsin has also been immobilized in monolithic supports and applied to the digestion of myoglobin ***(24).*** More recently, sol-gel

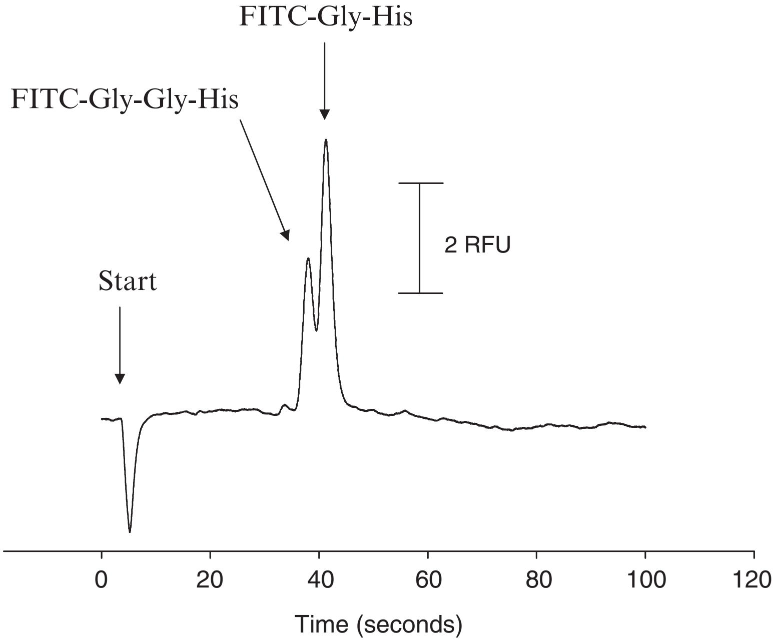

Fig. 2. Microchip CE separation of two-peptide mixture sampled on-line through 2-mm brain probe. (Parameters: 1.5 sec fill time, 2200 V). Analytes: FITC-Gly-His, 15 u*M*; FITC-Gly-Gly-His, 10 u*M*. Reprinted with permission from (19).

encapsulation has been investigated and used for the digestion of arginine ethyl ester (ArgOEt) and bradykinin ***(25, 26)*** (see ***Note 2***).

Fig. 3 shows a microchip device with integrated digestion, separation and postcolumn derivatization for the detection of labeled peptide fragments of insulin B-chain ***(27).*** More recently, an on-chip enzymatic reactor was demonstrated for rapid protein digestion ***(28).*** Protease was encapsulated in a silica sol-gel in a poly(ethylene terephthalate) PET microchannel. The digestion of cytoplasmic proteins from a human liver was achieved in the silica-gel based reactor within a few seconds.

Protein digestion followed by selective phosphopeptide enrichment has been demonstrated on a glass microchip device ***(29).*** The microchip consisted of two channels connected by a weir structure. The first channel contained agarose beads with immobilized trypsin while agarose beads with ferric ion were used in the second channel for immobilized metal ion affinity chromatography (IMAC). The device was also used for the enrichment of glycopeptides by replacing the IMAC beads with beads containing immobilized concanavalin A.

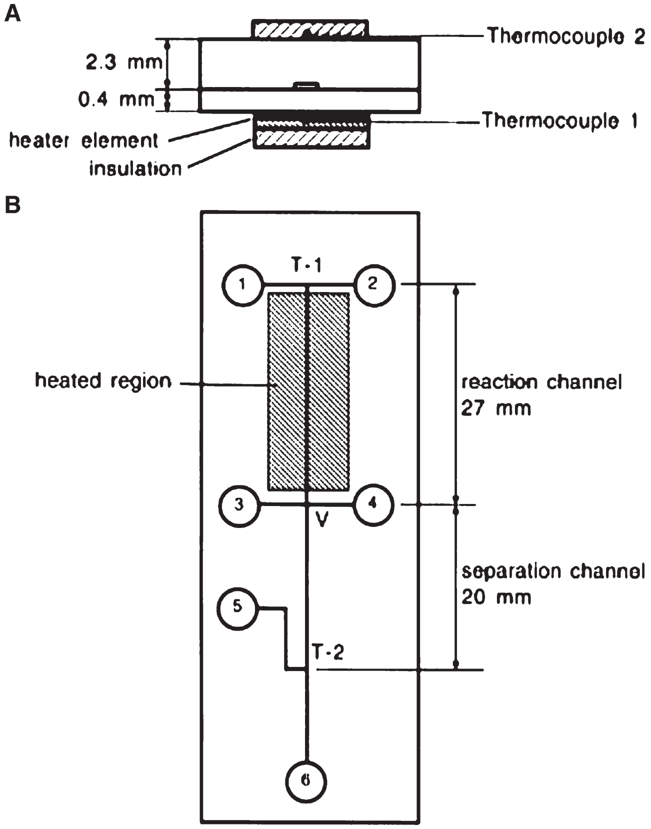
A
2.3 mm
0.4 mm
heater element
insulation
Thermocouple 2
Thermocouple 1
B
T-1
1
2
heated region
reaction channel
27 mm
3
4
V
separation channel
20 mm
5
T-2
6

Fig. 3. **(A)** Cross-sectional view of a CE microchip, heating element, and the thermocouples. **(B)** Schematic of the microchip used for on-chip reactions, incorporating separation and postcolumn labeling. The fluid reservoirs are: (1) substrate, (2) enzyme or DTT, (3) buffer, (4) sample waste, (5) NDA, and (6) waste. Reprinted with permission from *(27).*

2.2. Injection

Once sample cleanup has been performed there are a number of different options for loading into the separation channel. Generally, this necessitates voltage control over multiple reservoirs *(30).* Popular methods include pinched and gated injections.

Pinched injections demand simultaneous voltage control over all microchip reservoirs. A double T injector configuration reproducibly defines the volume of the sample plug. During separation, sample leakage into the channel is prevented by the application of a "pinching" or pushback voltage. Limitations of this approach include the need for multiple power supplies and the inability to change the volume injected using a double T design.

In gated injection schemes, the sample plug is not volume defined and can be altered. A voltage is applied to the sample reservoir with the sample waste reservoir held at ground. A second voltage is applied at the buffer reservoir with the buffer waste reservoir held at ground. Two separate flowing streams are established within the device. For injection, the sample reservoir is floated and a small plug of sample enters the separation channel. Both flows are then reestablished for separation. This approach uses a simple T injector configuration and requires two power supplies. While injection volumes can be varied, sample and buffer streams must be of similar ionic strength. Further information on injection methods is included in a recent review ***(31).***

2.3. Separation Methods

2.3.1. Capillary Zone Electrophoresis

Capillary zone electrophoresis (CZE) has been investigated for peptide separations on microchip systems due to the simplicity of the buffer system *(see **Note 3**)*. The separation of Gly-Leu and Gly-Gly on a glass microchip was demonstrated using a 20 m*M* borate buffer, pH 9.5 ***(32).*** A mixture of bradykinin, substance P, luteinizing hormone, bombasin, oxytocin, and enkephalins was separated on a PMMA device in 100 µM phosphate at pH 5.0 (33). A simple CZE buffer consisting of 15 m*M* boric acid, pH 9.2, was used for the separation of neuropeptides on PDMS microchip devices ***(34).*** Angiotensin peptides have been separated on both PDMS and glass microchip devices using 20 m*M* boric acid and 100 m*M* tris(hydroxymethyl)aminomethane, pH 9.0 *(7)*. **Fig. 4** shows separations of these peptides performed on both glass and PDMS microchips with the polymer devices displaying significantly lower separation efficiencies. This is most likely due to peptide adsorption to the channel surface *(see **Note 4**)***.** Methods to improve detection limits for microchip CZE of peptides include the use of polarity switching to initiate sample stacking ***(35).***

2.3.2. Micellar Electrokinetic Chromatography (MEKC)

In micellar electrokinetic chromatography (MEKC), separation is based on differences in the extent to which analytes partition between surfactant micelles and the surrounding aqueous buffer solution. When the concentration of a charged surfactant added to the buffer exceeds a value known as the critical micelle concentration (CMC), an aggregate, termed a micelle, is formed ***(36).*** The hydrophobic tails of the surfactant orient toward the center of the aggregate, and the polar headgroups orient toward the aqueous solution ***(37).*** A hydrophobic core is formed into which neutral hydrophobic analytes can partition ***(38).*** This method, initially developed for the separation of neutral molecules, is especially useful for peptides with a blocked N- or C-terminus. MEKC can also be used to separate charged peptides with similar electrophoretic mobilities but different

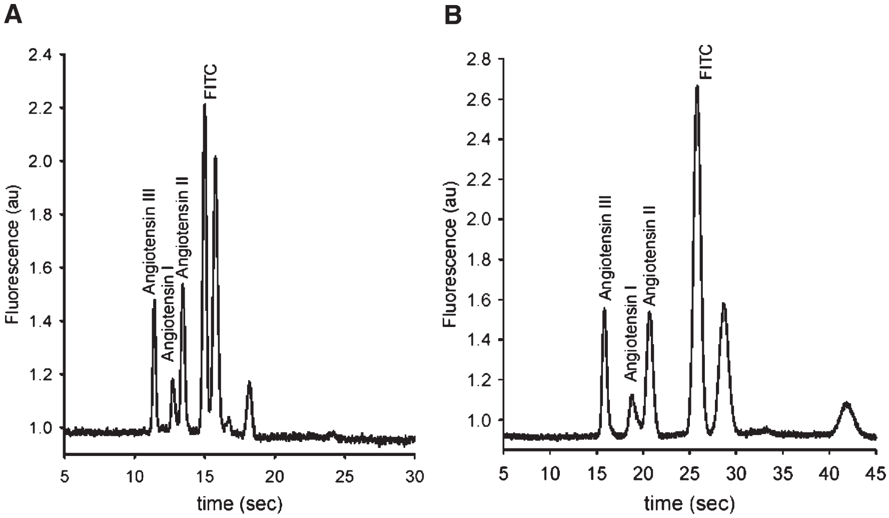

Fig. 4. Separation and laser induced fluorescence detection of FITC-labeled angiotensin peptides (angiotensin I, angiotensin II, and angiotensin III) at a concentration of 500 n*M* using an injection voltage of 2000 V. The buffer consisted of boric acid, Tris (20 m*M*, 100 m*M*, pH 9.0). **(A)** Separation obtained using a Pyrex microchip. The separation voltage used is 3200 V with an anti-leak voltage of 1600 V. **(B)** Separation obtained using a PDMS microchip. The separation voltage used is 4000 V with an antileak voltage of 2000 V. Reprinted with permission from ***(113).***

hydrophobicites. A microchip MEKC separation of lysozyme, trypsin inhibitor, and carbonic anyhydrase was accomplished on a PMMA microchip with 1% SDS in the TRIS-HCl run buffer ***(33).*** The separation of fluorescently labeled bovine serum albumin from other model proteins (cytochrome c, lysozyme, ribonculease A, myoglobin, and lactalbumin) in approximately 1.5 seconds on PDMS-based devices was accomplished by microchip-based MEKC ***(39).*** The separation efficiencies achieved were higher than those reported previously on native and modified PDMS microchip devices. MEKC has also been used in conjunction with 2-D separation schemes, which will be discussed later.

2.3.3. Isotachophoresis (ITP)

Isotachophoresis (ITP) is a separation technique based on electrophoresis occurring at a uniform speed where analyte migration is independent of mobility. It has been termed a "moving boundary" electrophoretic technique ***(40).*** ITP employs a leading and a terminating electrolyte with the sample zone sandwiched between them. For successful implementation of ITP, careful control of sample conductivity is needed. This can be difficult to obtain for biological samples containing high salt concentrations. In contrast to CZE, ITP will not separate anions and cations in the same run because EOF is generally suppressed.

For successful implementation of ITP, the leading electrolyte must have a greater mobility than any of the analytes in the sample, while the terminating electrolyte must have a lower mobility. When the electric field is generated, the ions of interest will migrate toward the electrode of opposite charge in zones determined by their electrophoretic mobility. Zone width adjusts so that all zones have same conductivity. After the initial zone formation, equilibrium is reached inside the channel, and the analyte ions then migrate past the detector at the same velocity. Narrowing of the analyte band can be accomplished by adding a high concentration of both the leading and terminating electrolyte.

Microchip-ITP has been used as a preconcentration technique to improve detection limits for the determination of fluorescently labeled peptide substrates following enzyme assay ***(41).*** Other applications have included the preconcentration of cytochrome c following on-line protein digestion ***(22)*** and characterization of peptide phosphorylation by protein kinase ***(42).***

2.3.4. Isoelectric Focusing

Isoelectric focusing (IEF) separates peptides and proteins based on differences in their isoelectric point (pI). IEF can be used to separate proteins differing in pI by 0.0005 units or less ***(40, 43–46).*** An advantage of IEF is that the whole channel can be filled with sample, improving detection sensitivity. The sample is dissolved in a mixture of carrier ampholytes prepared in different pH ranges, and the channel is filled ***(47).*** Loading is followed by focusing and mobilization steps (**Fig. 5**).

Once the sample has been loaded, a voltage is applied and the carrier ampholytes separate into individual bands, establishing a pH gradient inside of the microchip. There are three different types of behavior that the peptide can exhibit once the voltage is applied. If the pH of the band is lower than the pI, the peptide will be positively charged and will migrate toward the cathode. If the pH is higher than the pI, the peptide will be negatively charged and will migrate toward the anode and, if the pI is equal to the pH, the peptide is neutral and will not migrate. All peptides will migrate until the pH is equal to the pI. At this point the current will approach zero, the resistance in the channel will be high, and focusing will cease. Eventually, all of the peptides will be focused into bands where their pI is equal to the pH and they will stop migrating. One drawback of IEF is that, because there is a pH gradient formed along the channel, the extent of surface ionization varies. This can produce an uneven generation of EOF and cause band broadening. Therefore, the channel is usually modified to eliminate the EOF and to ensure that focusing is achieved prior to detection. This is accomplished by coating the microchip walls with a neutral coating, which also limits peptide adsorption ***(48).***

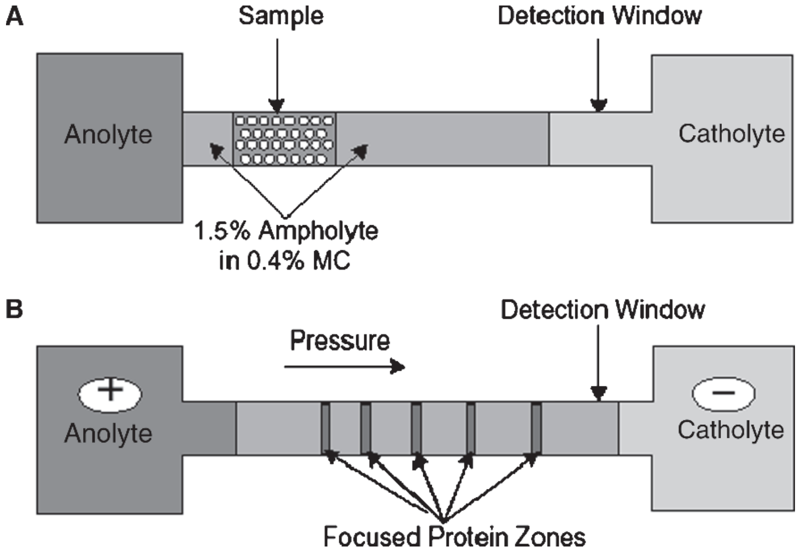

Fig. 5. Principle of capillary isoelectric focusing by simultaneous pressure/voltage mobilization. **(A)** Catholyte is backflushed past the detection point and a sample plug is introduced into the coated capillary (no high voltage). **(B)** Focusing of sample is complete and the sample components are driven toward the detector by a low-pressure rinse. High voltage is applied during this step. Reprinted with permission from ***(47).***

For single point detection systems, analytes must be mobilized past the detector region following the focusing step. The two most common approaches to analyte mobilization are hydraulic or chemical ***(37).*** Hydraulic mobilization of the focused zones occurs by applying either pressure or a vacuum to one end of a channel. Chemical mobilization is accomplished through the addition of a salt, such as NaCl, to one of the buffer reservoirs, followed by application of a high voltage. The actual mobilization in this case occurs due to the presence of a competing ion such as Cl^- that competes with OH^-. This lowers the pH, and the peptides become charged and migrate toward the cathode. The Na^+ competes with H^+, causing an increase in pH and making more negative analytes migrate toward the anode. This pH shift occurs across the entire length of the microchip channel ***(37).*** The end result of this pH shift is the mobilization of the previously focused analytes toward the cathode.

An alternative approach termed "one-step IEF" achieves focusing and mobilization simultaneously. As the pH gradient is not stationary, the EOF mobilizes the sample zones during focusing. One-step IEF can be accomplished using either coated or uncoated devices ***(49).*** EOF-driven mobilization is useful for microchip systems due to the speed of analysis and minimal instrumentation requirements. This approach was used for the separation of Cy5-labeled peptides; however, higher separation efficiencies and reproducibilities were achieved using chemical and hydraulic mobilization strategies ***(49).***

The requirement for sample mobilization past a single detection point can be obviated through the use of whole column imaging. This technique has been applied to the separation of test peptides using a UV absorbance detector ***(50).*** Due to reduced detection sensitivity, high concentrations of peptide were required, and glycerol was added to maintain peptide solubility. A more sensitive approach used fluorescence detection for microchip IEF of rhodamine green-labeled peptides on a PMMA device ***(51).*** Real-time imaging of the channel was performed using a scanning detection system that facilitated the determination of peptide migration times along with the estimation of EOF and pressure-driven flows. A scanning detection system based on acousto-optical deflection has also been developed and used for imaging of proteins during IEF ***(52).*** Advantages of this technology include fast response times, the absence of mechanical parts, and minimal background noise. It is also possible to perform on-chip mobilization with an on-chip diaphragm following isoelectrofocusing in the microchip channel ***(53).***

2.3.5. Electrochromatography

The development of capillary electrochromatography (CEC), a hybrid of CE and liquid chromatography (LC), has allowed highly efficient separations of peptide mixtures ***(54).*** In CEC, the channel is packed with a stationary phase for chromatographic analyte retention similar to LC, but electroosmotic flow is also generated. The main advantage of this technique over LC is improved peak efficiencies due to the flat plug flow profile generated by the EOF ***(48).***

The use of conventional bead-based retention systems in microfluidic devices requires methods (weirs, channels) to localize the beads and prevent device clogging. A 200-µm channel packed with octadecylsilyl (ODS) stationary phase particles was used to separate a fluorescently labeled angiotensin peptide from excess labeling agent ***(55).***

In chip-based electrochromatography, the stationary phase, the channel, inlets, and outlets can all be produced in one device using lithographic patterning techniques that are already employed for the fabrication of microfluidic devices. Collocated monolithic support structures (COMOSS) that are molded directly into the channel have been created in both quartz and PDMS ***(56, 57).*** Advantages of this approach include a uniform distribution of support structures defined during fabrication and homogenous channel dimensions. Issues involved with packing and retention of stationary phase particles are also eliminated as the reversed-phase stationary phase is bonded directly to the structures, and there is no need for the production of frits. **Fig. 6** shows a COMOSS fabricated in PDMS. Applications of COMOSS modified with C18 phases include the

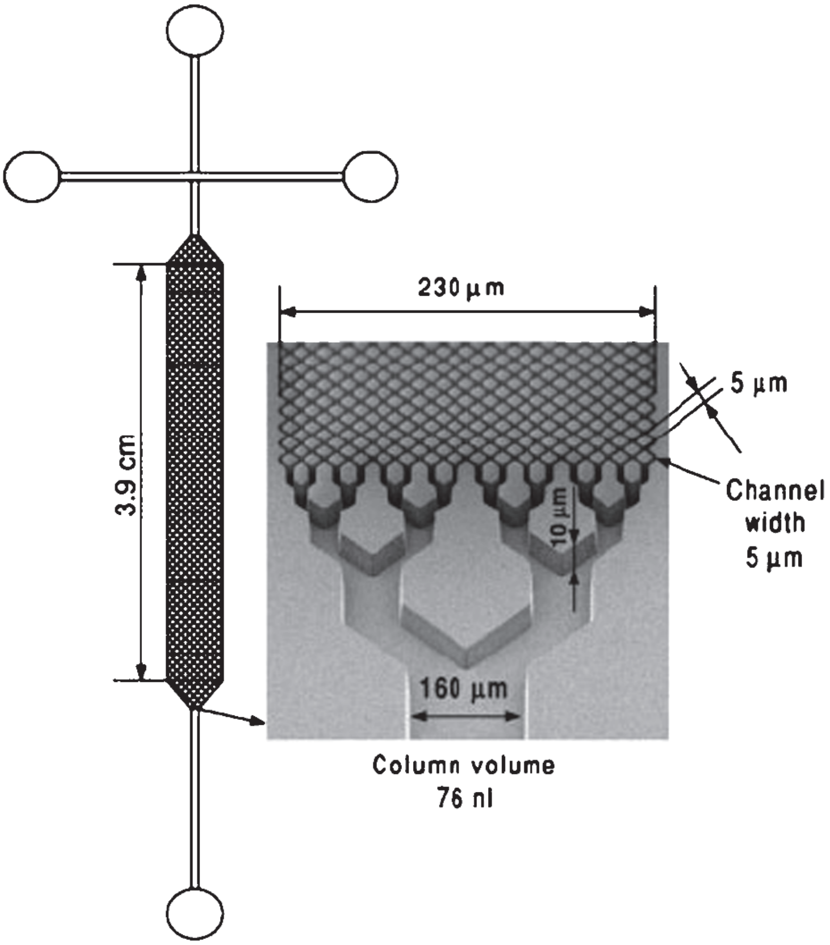

Fig. 6. Scheme of a COMOSS separation column. Reprinted with permission from ***(57).***

separation of tryptic digests of ovalbumin ***(56)*** and fluoroisothiocyanate-labeled bovine serum albumin ***(58).***

Monolithic polymer stationary phase materials are proving useful for microchip CEC applications **(Fig. 7)**. Advantages of these phases include *in situ* polymerization, high flow rate tolerance, and functionalities that can be tailored to suit a specific application (59). In situ photopolymerization can be achieved by selective exposure to UV light. The location of the polymer can be accurately defined using a photomask. Acrylate-based porous polymers have been cast in glass channels for the separation of fluorescently labeled peptides, including angiotensins ***(60).*** The glass microchips could be reused following thermal incineration of the monolith at 550°C for 2 h and overnight incubation in 0.2 N NaOH.

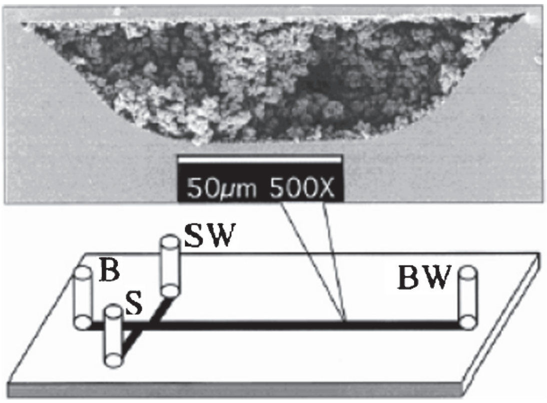

Fig. 7. Schematic of the microchip used for electrochromatography. B, S, BW, and SW denote reservoirs containing buffer, sample, buffer waste, and sample waste, respectively. The inset shows a scanning electron micrograph (SEM) of a channel cross section filled with photoinitiated acrylate polmer monolith. The mean pore diameter is 1 mm. Reprinted with permission from ***(60).***

Sol-gels modified with polyelectrolyte multilayers are also being investigated for potential application as cation-exchange materials for microchip CEC separations of peptides including enkephalins ***(61).***

2.3.6. Electrophoretic Bioaffinity Assays

Electrophoretic bioaffinity assays combine the specificity of an immunological or enzymatic response with the separation power of electrophoresis. Separation and quantification of enzymatic conversion or antibody/antigen binding can be achieved using microchip CE. Potential applications of affinity separation technologies include biochemical, clinical, and drug development operations ***(62).***

2.3.6.1. IMMUNOASSAYS

Immunoassay techniques are employed for the determination of binding constants for antibodies and antigens in competitive and noncompetitive studies. The separation of human IgM and antihuman immunoglobulin *M* has been achieved using a PMMA microchip device with conductivity detection ***(63).*** Tween surfactant was added to the run buffer to reduce the adsorption of the proteins to the channel wall. A PMMA device was also used for the separation of fluorescently labeled human anti-goat antibody and unlabeled goat immunoglobulin G (IgG). In this case, no significant adsorption of IgG protein was observed ***(64).***

MEKC has been employed for an immunoassay monitoring serum levels of theophylline ***(65).*** A competitive immunoassay to monitor theophylline in serum samples and a direct assay for the determination of monoclonal mouse immunoglobulin G in mouse ascites fluid were demonstrated on-chip using a tricine buffer containing Tween 20 and NaCl ***(66).*** A subsequent microchip system incorporating mixing, reaction, separation, and detection steps was developed for the same application ***(67).***

A microchip CE device that performed electrokinetic mixing of antibody to bovine serum albumin (BSA) and a diluting buffer was demonstrated for the on-chip preparation of calibration standards. These standards were then reacted with flourescein-labeled BSA, allowing the on-line generation of a standard curve. The device was used to assay the BSA antibody prepared from diluted mouse ascites fluid ***(68).***

A competitive chip-based immunoassay has been developed for the determination of insulin secretion from single islet cells ***(69).*** Online mixing of reagents allowed continuous sampling, and the simple device design needed only one voltage power supply for operation. A competitive immunoassay using rabbit polyclonal anti-cortisol antiserum and a fluorescently labeled cortisol derivative was demonstrated on a glass microchip device ***(70).*** The working range of the device was determined to be within clinical range (1–60 µg/dL cortisol).

A heterogeneous competitive immunoassay of human immunoglobulin G (IgG) was demonstrated on a hybrid PDMS and glass device ***(71).*** Cy5-labeled human IgG was used as a tracer and Cy3-mouse IgG was used as internal standard. The microchip system was used for the determination of IgG levels in human serum with fluorescence detection. The determination of binding efficiencies between diketopiperazine receptors and Arg-containing peptides was demonstrated on a commercial Shimadzu microchip system ***(72).*** The separation times achieved with the microchip system were 50 times shorter than what could be achieved on a conventional system.

2.3.6.2. Enzyme Assays

The determination of endogenous extracellular signal-regulated protein kinase was achieved using microchip CE to separate the substrate and product of the enzyme assay from an internal standard within 20 s. The device was used to measure the activity of endogenous levels of the enzyme in cell lysates ***(73).*** **Fig. 8** shows a separation of unphosphorylated and phosphorylated fluorescein-labeled "Kemptide" following on-chip enymatic conversion in a reagent well by protein kinase ***(74).*** The Km for "Kemptide" was determined using Lineweaver-Burk plots.

A capillary array was used to demonstrate a multiplexed approach to the analysis of kinases ***(75).*** The assay was performed by the addition of multiple enzymes to a reaction tube. Simultaneous resolution of four product and three substrate peaks was demonstrated within 30 s. The multiplexed approach was

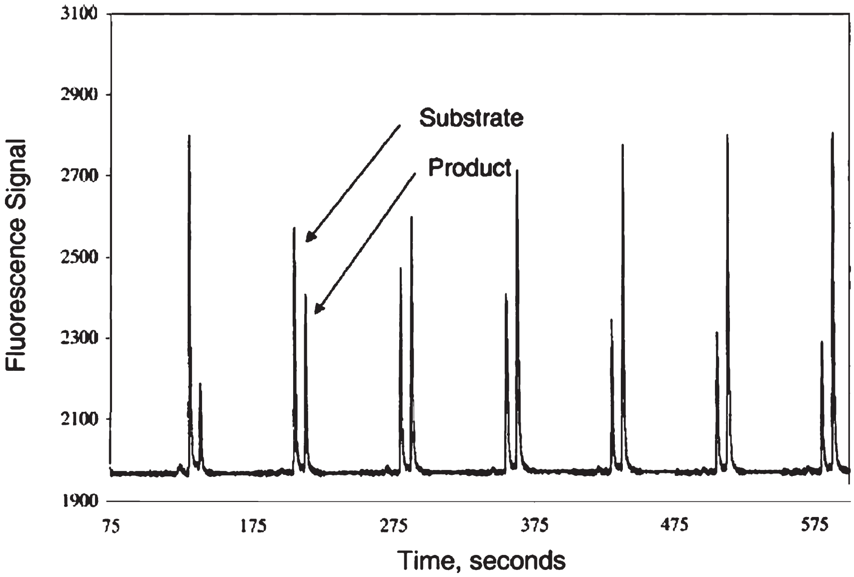

Fig. 8. Time course of the Protein Kinase A (PKA) reaction on a 12A chip. Electrophoretic separation of unphosphorylated and phosphorylated fluorescein labeled Kemptide upon enzymatic conversion by PKA in a reagent well of the 12A microchip. Conditions: 13.3 m*M* Fl-Kemptide, 24.5 m*M* PKA in 100 m*M* Hepes, pH 7.5, 1 *M* NDSB-195, 5 m*M* $MgCl_2$, 100 m*M* ATP, 50 m*M* cAMP, 0.1% Triton X-100, 10 m*M* DTT. Reprinted with permission from *(74)*.

also tested using a phospholipase and another kinase, demonstrating the ability to screen different types of enzymes and potential inhibitors in one analysis.

Another advantage of microchip systems is the ability to fabricate multiple channels for high throughput analysis. Microchip array electrophoresis has also been used to monitor the kinetics of a phosphate substrate and alkaline phosphatase conjugate as a function of reaction time *(76)*. The enzymatic products were detected using laser-induced fluorescence with a miniature semiconductor laser. An electrochemical enzyme immunoassay was developed incorporating post-column reactions of alkaline phosphatase-labeled antibody *(77)*. Amperometric detection was then used to identify the free antibody and the antibody-antigen complex.

Microchip CE has been used for the separation of four different inhibitors of acetylcholinesterase following on-chip mixing of enzyme, substrate, inhibitor and derivatizing reagents *(78)*. Acetylthiocholine is hydrolyzed to thiocholine by acetylcholinesterase. The reaction of thiocholine and coumarinylphenylmaleimide forms a thioether that can be detected using fluorescence. Modulation of enzyme activity was identified by a decrease in fluorescence intensity.

2.3.7. Multidimensional separations

Chromatographic and electrophoretic separation systems have a maximum peak capacity that is dependent on column length and separation efficiency. With LC the peak capacity typically ranges from 20 to 100, while for CE values of 50–100 can be obtained ***(79).*** For the analysis of complex biological samples, a one-dimensional system will have a limited peak capacity; therefore, the use of two-dimensional separations can extend the applicability of the technique. For 2-D separations, the output of the first separation technique serves as the input to the second. Therefore to successfully analyze each peak, the separation in the second dimension must be faster than in the first.

A conventional capillary HPLC system has been interfaced to microchip electrophoresis for the separation of fluorescently labeled peptides from tryptic digests of bovine serum albumin ***(80).*** Effluent from the HPLC was injected into the CE channel every 20 s. On- chip coupling of 2-D separation systems is facilitated by the planar nature of the microchip device. A combination of MEKC (10 m*M* sodium dodecyl sulfate) and CZE was demonstrated on a microchip platform for the separation of tryptic peptides (79). Output from the first dimension was introduced to the second using a gated injection scheme. A similar approach was adopted for the coupling of open channel electrochromatography and CE on a glass microchip ***(81).*** **Fig. 9a** shows the device used with the spiral channel employed for the CEC, which was coated with a C18 phase connected

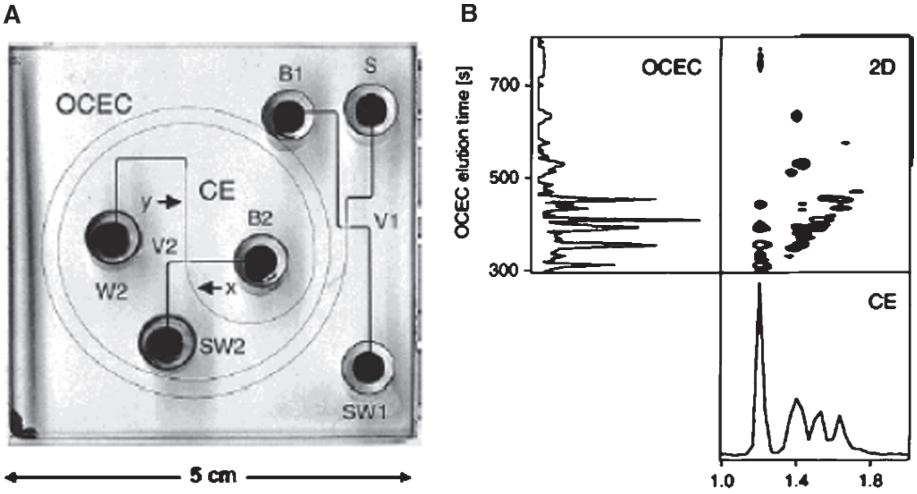

Fig. 9. CEC-CE chip. **(A)** The CEC serpentine channel extends from V1 to V2 (25 cm), the CE channel from V2 to the detection point y (0.8 cm), **(B)** 2-D contour plot of a β-casein tryptic digest. Reprinted with permission from ***(81).***

to the straight channel employed for CZE in the second dimension. **Fig. 9b** shows the application of the device to a 2-D separation of fluorescently labeled tryptic peptides of β-caesin.

Acrylic devices have been employed for the on-chip coupling of IEF and CZE ***(82).*** The acrylic microchip supported a reduced EOF relative to that of glass, and this property was exploited as a slow mobilization method. When the output of the first dimension (IEF channel) is introduced to the second (CZE channel), the voltage is switched and applied to the CE channel only. As a result, the analysis time for the entire system is dominated by that of the second dimension (<1 min). The device was applied to the analysis of fluorescently labeled dextran and ovalbumin.

On-chip tryptic digestion followed by 2-D electrochromatography has been demonstrated for histidine-containing peptides on a PDMS device ***(83).*** Digestion of bovine serum albumin was followed by separation of the peptides by metal affinity chromatography and reversed-phase electrochromatography.

2.4. Detection modes

Once a separation has been achieved, the most popular modes for peptide detection include fluorescence, electrochemistry (EC), or mass spectrometry (MS). Due to enhanced sensitivity, fluorescence and MS are the more popular modes of detection for peptide analysis with microchip CE.

2.4.1. Fluorescence detection

Fluorescence detection is the most commonly reported detection mode for microchip CE due the high sensitivity that can be obtained and the ability to focus collimated light onto micron-sized separation channels with relative ease. Excitation sources include lamps and LEDs; however, despite their high cost, lasers are also popular due to their ability to provide high intensity radiation.

Some peptides and proteins contain fluorescent amino acids such as tyrosine and tryptophan and exhibit native fluorescence ***(84).*** Recent advances in laser technologies may facilitate the on-chip detection of peptides by native fluorescence in the near future. However, in most cases, peptides and proteins must be labeled with an extrinsic fluorophore prior to analysis in order to be detected. Fluorescein-5-isothiocyanate (FITC) is the most popular reagent used for labeling of peptides; it is compatible with the 488 nm line of the argon-ion laser. Other fluorophores that have been employed include fluorescein, o-phthaldialdehyde (OPA), naphthalene-2,3-dicarboxaldehyde (NDA), BODIPY® TR, rhodamine red, dansyl chloride, 5- (and 6-) carboxyfluorescein, and NanoOrange. Cy5 label has been used to label proteins through the primary amines. Cy5 is excited at 633 nm, with collection of resulting emission at 680 nm.

The reaction rate, fluorescent product stability, quantum yield, excitation wavelength, and emission wavelength should all be considered when selecting a reagent for a specific application. On-chip derivatization can take place prior to analysis (precolumn) or after the separation (postcolumn). The on-line post-column labeling of peptide fragments following tryptic digestion of oxidized insulin B-chain has been demonstrated using NDA/CN ***(27).***

2.4.2. Electrochemical detection

Electrochemistry is currently the detection mode most amenable to the production of a truly portable device. As the response at the electrode is not pathlength dependent, electrochemical detectors can be miniaturized without a loss in sensitivity. Electrodes can be microfabricated using the same techniques employed for chip manufacture and integrated onto the microchip platform. While EC detection encompasses conductimetry, potentiometry, and amperometry, the latter is the most commonly reported mode of EC detection for microchip-based peptide separations ***(31, 85–91).***

Electroactive amino acids include tyrosine (Tyr), tryptophan (Trp), and cysteine (Cys). If the peptide does not contain one of these electroactive amino acids, it must then be derivatized or complexed with an electroactive substance in order to enable detection. Derivatization agents that label the primary amine of the peptide have been used to enable electrochemical detection of amine-containing compounds; these include o-phthaldialdehyde/β-mercaptoethanol (OPA/βME) and naphthalene-2,3dicarbox-aldehyde/cyanide (NDA/CN). Both are oxidized at + 750 mV vs. Ag/AgCl at a carbon electrode ***(90, 92).*** Precolumn derivatization of small peptides with NDA/CN for electrochemical detection was demonstrated on a glass microchip device using a reaction chamber ***(32).*** An end-column detection alignment was used in conjunction with an external screen-printed carbon electrode *(see* ***Note 5****)*.

Most schemes require that the analyte possess a primary amine functionality for derivatization; however, it is possible to detect peptides with blocked N-termini, peptides lacking lysine, and cyclic peptides by the formation of a peptide-copper (II) complex ***(93, 94).*** As these complexes can undergo reversible redox reactions, dual-electrode detection can be used. This approach typically involves oxidation at the first electrode and reduction at the second. **Fig. 10** shows a dual-electrode electropherogram of the Des-Tyr Leu-enkephalin copper complex obtained using a microchip CE-EC device **(88).** A mixture of 50 m*M* boric acid, 3 m*M* tartaric acid, and 1 m*M* $CuSO_4$ buffered at pH 9.8 was used for separation ***(see note 6).***

While amperometry has proven to be the most popular mode of electrochemical detection for peptides, several examples of the use of conductivity detection have also been described. Conductivity detection is a universal detection technique that can be used in conjunction with microchip CE. The only requirement is that the conductivity of the analyte is different from that of the carrier electrolyte. An integrated conductivity detector for microchip CE has been described ***(33).*** The detector was constructed from platinum wires aligned using guide channels

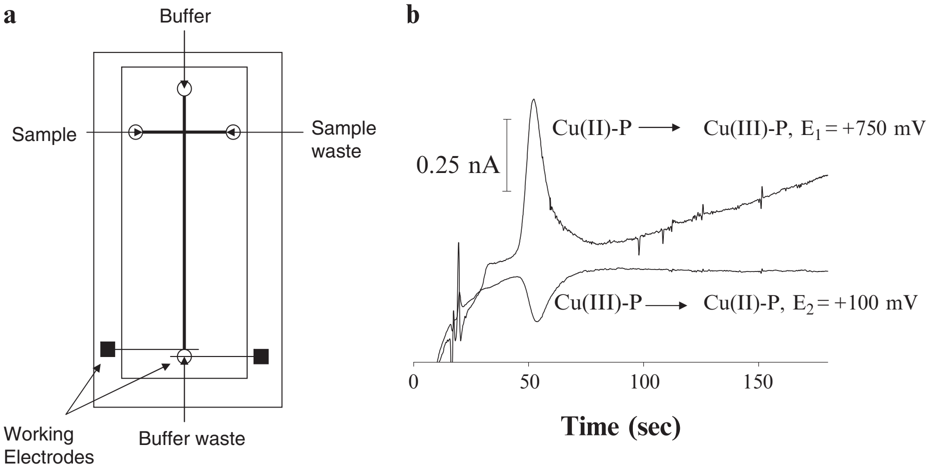

Fig. 10. **(A)** Microchip CE with end-column dual electrode detection. **(B)** Separation and dual-electrode detection in a series configuration with carbon paste electrodes. Detection of precomplexed copper complexes of: TyrGlyGly (340 µ*M*) and des-Tyr leu-enkephalin (450 µ*M*). Separation conditions: 50 m*M* boric acid, 3 m*M* tartaric acid, 1 m*M* copper sulfate, pH 9.8; applied voltage = 1170 V (350 V/cm). Injection: 1 second (S to SW) at 1170 V. E1 = +900 mV vs. Ag/AgCl; E2 = +300 mV vs. Ag/AgCl. Reprinted with permission from ***(88).***

and integrated into a PMMA microchip device by the thermal annealing of a PMMA cover plate. A CZE separation with indirect detection of a mixture of nine peptides, including bradykinin, substance P, and leu-enkephalin, was demonstrated. The same device and configuration were applied to the separation of a protein mixture by MEKC.

While the simultaneous detection of multiple targets is a feature of any separation-based system, a key advantage of microchip platforms is the potential to multiplex the analysis through the use of parallel processing. A 16-channel chip system that combines an integrated conductivity sensor array has been fabricated using a polycarbonate substrate ***(95).*** Each channel was addressed individually by a pair of detection electrodes incorporated during the fabrication of the device. High-speed parallel separations of amino acids, peptides, proteins and oligonucleotides were achieved using both CZE and CEC. The microchip reservoirs were designed to the same pitch as a multipipettor, thus allowing rapid loading of samples and regents to the chip.

A limitation of contact conductivity detection is the lower field strengths that must be applied (generally in the order of 50 V/cm) to minimize gas generation and bubble formation at the electrodes. This can influence separation efficiency but can be overcome through the use of a contactless approach. As there is no direct contact with the solution, electrode fouling is no longer an issue. This approach was used in conjunction with glass microchips for the detection of two small test peptides. Capacitive coupling with the separation electrolyte was achieved by coating electrodes with a thin layer of silicon carbide, that acted as the insulating layer ***(96).*** A contactless configuration was also employed for the detection of human immunoglobulin *M* in glass and PMMA microchip devices ***(63).*** Tween surfactant, added to the buffer to minimize adsorption to the walls of the device, did not appear to interfere with the detection.

2.4.3. Mass Spectrometric Detection

Due to the ability of mass spectrometry to provide molecular weight and structural information without the need for derivatization of analytes, it has become a very desirable detection mode for peptides. Mass spectrometry is also useful for high throughput analysis. The ability to make disposable separation devices obviates many of the issues of cross contamination, a concern for high throughput applications. Often, microfluidic chips are used for sample introduction into the MS as the low flow rates used are compatible with sample delivery. The ability to integrate sample cleanup prior to analysis is also an advantage.

Electrospray ionization (ESI) is the most popular ionization technique used in conjunction with microfabricated devices, although matrix–assisted laser desorption/ionization is also compatible ***(97–99).*** Ionization can occur on– or off–chip, with some emitters fabricated directly onto the chip device ***(97, 100).*** For successful ionization, analytes must be charged and in solution. Ions are transferred to the gas phase by generation of an electric field between two electrodes, forming a spray of sample. ESI is compatible with a number of mass analyzers; however, time-of-flight and quadrupole-time-of-flight mass analyzers are perhaps best suited to handling high throughput analyses and have found greater application.

Considerations for coupling microchip to MS include minimization of dead-volume, establishing a steady flow rate for sample introduction, minimizing sample adsorption to the microfluidic device, and solvent compatibility of the device. In addition careful selection of the pH of the analyte solution is required, as this will determine analyte charge (this relates to the detection mode setting for positive or negative ion monitoring), and the magnitude and direction of EOF generated on the microchip device (this will determine whether the peptide mobility is sufficient to reach the emitter) ***(101).***

A microfabricated chip was interfaced to ESI-MS and applied to the analysis of a tryptic digest of β-caesin ***(102).*** Coating the microchannel and the transfer capillary with a polybrene solution improved both delivery and response. The separation and identification of peptides were achieved using a microfabricated CE device with nanoelectrospray mass spectrometry ***(103).*** Initial protein extraction was followed by SDS-PAGE and tryptic digestion. The microfluidic device was then used to perform sample cleanup prior to mass spectrometric analysis.

A microfluidic device has also been coupled to a quadrupole TOF-MS for trace analysis of protein digests isolated on a gel. Sample stacking was used to improve detection limits for hydrophilic peptides while solid-phase extraction techniques were used to preconcentrate hydrophobic peptides ***(35).***

Sample preparation procedures such as filtration, solid phase extraction (SPE), preconcentration, and protein digestion have been incorporated onto the chip platform prior to the introduction of the sample into the mass spectrometer representing some of the highest degrees of integration ***(104–107***). Analysis of both proteins and peptides has been achieved using this approach ***(108).*** Further optimization of chip-to-MS interfaces combining ionization and de-salting steps is needed before this technology will be used on a routine basis.

Many groups are now attempting to fabricate the tips for electrospray as part of the fluidic device. This approach allows easier coupling of high density devices directly to MS detection systems. A micro-nib interface for NanoESI-MS has been developed for microfluidic applications ***(109).*** The fabrication of a planar nib structure was achieved using SU-8 and applied to an ion trap mass spectrometer for the analysis of a standard peptide solution of Gramicidin S down to 1 μM. The same group has also implemented a polysilicon nanoelectrospray-mass spectrometry source ***(110).*** The source is based on a planar low-stress triangular polysilicon cantilever that projects horizontally from a silicon wafer to form a nib-like structure. Detection of a standard peptide sample of Glu-Fibrinopeptide B was achieved.

An integrated system for the preparation of a protein sample followed by online analysis by ESI-MS has also been reported ***(111).*** The system is fabricated in SU8 and contains a digestion module and monolithic phase material for chromatographic separation. Coupling of the microchannel outlet to ESI-MS through a nib-shaped outlet interface allowed the detection of a standard peptide mixture.

Another highly integrated approach to peptide analysis incorporating 2-D solid phase extraction capillary electrophoresis for ESI-TOF-MS has been described ***(112).*** The PDMS microchannel device comprised two channels; one for sample introduction and desalting and the other for CE separation. The desalting was achieved using 5 μm polystyrene beads. The channels were modified with a positively charged polymer PolyE-323 to reduce the adsorption of the peptide mixtures. A sample solution of six neuropeptides was desalted, separated, and sprayed into a TOF-MS for detection.

3. Summary

Microchip electrophoresis has made great strides since the initial concept was described by Manz and coworkers in 1990 ***(5).*** Thus far, CZE has been commonly employed as separation method on-chip, but other CE modes such as MEKC, CEC, ITP, IEF, and 2-D separations are being applied to peptide analysis. Many of the detection methods used with conventional systems, such as LIF, EC, and MS, have been successfully adapted for use with microchip systems. Recent advances in microfabrication and packaging techniques have facilitated the production of highly integrated devices capable of parallel processing for high throughput analysis and the incorporation of nib-like structures for easier and reproducible interface to mass spectrometers. The continued development of truly integrated miniaturized analysis systems that can perform sample isolation, digestion, separation, and detection will significantly improve the field of peptide analysis. The resulting devices could then be used for screening, cell culture, and proteomic studies.

4. Notes

1. Selection of the microchip substrate will ultimately depend on compatibility with sample and buffer solutions and fabrication facilities available. Glass microchips are fabricated using chemical etch procedures whereas polymer devices are typically fabricated by hot embossing or casting techniques. Due to the hydrophobic nature of the polymer PDMS, reversible sealing of the separate layers can be achieved through contact bonding. If a permanent seal is required, PDMS may be plasma oxidized, which also improves the magnitude of EOF over that of native devices.
2. For on-chip protein digestion, immobilization of trypsin in sol-gel materials can increase stability compared to that in free solution.
3. For simple CZE separations, microchip channels should be conditioned at the beginning of the day by sequentially flushing for 10 min with degassed and filtered solutions of 0.1 *M* NaOH, H_2O, and buffer. At the end of the day, channels should be rinsed with water to prevent buffer crystal precipitation.
4. The use of acidic or basic buffer pHs can aid charge repulsion from channel surfaces minimizing peptide adsorption. Neutral hydrophilic coatings can help to reduce issues of peptide adsorption to channel walls. For CEC applications, using an acidic elution buffer can reduce peptide adsorption and tailing effects, commonly seen with silica-based phases.
5. A major consideration for EC detection with any CE application is the isolation of the separation field from the detector to protect the potentiostat. For amperometric detection, electrodes can be aligned at the end of the channel to protect the poten-tiostat from the high field strengths needed for the electrophoretic separation. However, limitations of this approach include analyte diffusion, leading to reduced peak efficiencies. Alternative approaches to the effective isolation of the detector from the separation voltage include off-channel detection using decouplers designed to shunt away separation voltage prior to detection or in-channel detection requiring the development of electronically isolated (floating) potentiostats.

6. Copper(II)-peptide complexes oxidize at a carbon electrode (+750 mV) vs. an Ag/AgCl reference. For dual detection the Cu(II) complex is oxidized at the first electrode and the resulting Cu(III) complex is reduced to a Cu(II) complex at the second electrode. The reduction of the complex at the second electrode takes place at a mild reduction potential (+100 to +200 mV), which results in a better S/N ratio, lower LOD, and better selectivity.

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38

Protein Separations in Microfluidic Chips

Andrea W. Chow and Bahram Fathollahi

1. Introduction

Identifying protein structure, function, and expression level under varying environmental conditions in cells is an important activity in most life science research and pharmaceutical discovery. Determining the molecular weight and purity of protein samples is routinely done in laboratories using sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) in the last few decades ***(1, 2).*** This process is highly manual and takes many hours to prepare the gel, run the samples, stain and destain the protein fractions, capture the gel image, and analyze the image to determine the protein sizes against a ladder. With commercially available precast gel, the first step of the process is becoming simpler and less messy, but overall it is still a relatively slow and tedious procedure. In large-scale protein processing operations and proteomics research laboratories, protein analysis often is a bottleneck in the workflow. Over the last 10 years capillary gel electrophoresis-SDS (SDS-CGE) is being increasingly used for analysis of therapeutic proteins. Compared to SDS-PAGE, SDS-CGE offers advantages of on-column detection, improved quantitation and resolution, and automation. However, SDS-CGE is limited by low sensitivity, throughput, and reliability. In the past several years, microfluidic-based assay for sizing, quantitiation, and purity assessment of proteins are also finding rapid adoption. The benefits in integration, fast separation, automation, and ease of use offered by microfluidics address the limitations of SDS-PAGE and SDS-CGE, and have proven to be a faster and easier alternative to the traditional SDS-PAGE or SDS-CGE.

In microchip devices, gel electrophoresis can be performed in a much shorter time scale, typically by more than an order of magnitude improvement, than in capillaries and slab gels with equivalent performance metrics such as the number of theoretical plates and resolution. The main reason for the faster separation is the ability to inject a much narrower band of sample in a microfluidic

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device, typically on the order of 10–100 µm. In electrophoretic separation, the number of theoretical plates *(N)* increases as the square of column length *(L)* for small sample plug:

$$N = 12\,(L/w)^2/(1+L/L_0);$$
$$L_0 = \mu E w^2/24D$$

where μ is the electrophoretic mobility of the analyte, E is the electric field, D is the diffusion coefficient of the analyte, and w is the root mean square of the width of the sample band and the width of the detector. In microfluidic experiments, the detector is typically focused down to 10–50 µm where it becomes comparable in length scale to the sample width. The time needed to achieve certain degree of resolution between peaks (for $L=L_0$) scales strongly with w:

$$t_0 = w^2/24D$$

A reduction of "w" by 10-fold results in a reduction of separation time by 100-fold. As a result of the faster separation time, the requirement for column length L has also been reduced from tens of centimeters in capillaries and slab gels to merely a few centimeters in a microchip.

When a protein sample is denatured in the presence of a high concentration of SDS above its critical micellar concentration (CMC), the protein molecules are coated by SDS micelles, in general in the ratio of about 1.4 g of SDS per g of protein ***(3)***. This binding ratio, somewhat surprisingly, is relatively independent of the protein sequence for proteins above 15 kDa. As a result of this interaction, the native charge of the protein is mostly masked by the charge of SDS, and the overall charge of the protein-SDS complex is primarily determined by the number of SDS micelles associating to the protein molecule. In the presence of an electric field without a polymer sizing matrix such as a polyacryamide gel, all protein-SDS complex species migrate electrophoretically at roughly the same speed regardless of protein size, because the charge of the complex increases proportionately with the protein molecular weight. In the presence of a sizing matrix, on the other hand, the differential hydrodynamic interaction of the complex with the matrix causes the larger complexes to migrate slower than the smaller ones, resulting in an effective sizing mechanism by electrophoresis.

The implementation of sizing protein-SDS complexes in capillary electrophoresis (CE) has not been widely adopted, unlike in sizing of DNA using CE. The difficulty comes from the lack of a convenient and sensitive means to detect the separated protein species. For DNA, a selection of fluorescent intercalation dyes binding to the double stranded DNA structure is available to detect the separated DNA fragments in CE with high sensitivity. For proteins, however, fluorescent dyes are available to stain the SDS micelles bound to the protein molecules, but they also stain the free SDS micelles in the gel, resulting in a low signal-to-noise protein measurement due to the high fluorescent background caused by the signal from the free micelles.

This high fluorescent background problem has been cleverly solved in a microfluidic chip using an integrated on-chip “destaining” step ***(4).*** After the protein fractions are sufficiently resolved in the separation column, an intersecting channel structure is introduced as shown in **Fig. 1.** The side channels are filled with the same polymer sizing matrix as in the main separation channel, but the buffer solution contains no SDS. By applying an electric current from the side channels into the main channel, two focusing streams containing no SDS are induced, creating a concentration gradient in SDS transverse to the flow direction downstream of the intersection. By Brownian diffusion, free SDS micelles in the center of the channel diffuse laterally outward into the zones of low SDS concentration, causing a breakup of the micellar structure when the local concentration of SDS drops below CMC. As free micelles break up, fluorescent dyes are released. Using dyes that do not fluoresce when they are dissociated from the micelles, the high background drops to a low level. This destaining process inside the microchannel network is photographically captured in the right-hand side in **Fig. 1.** Bound SDS micelles, on the other hand, cannot migrate outward as readily due to the large protein size and they tend to remain stable due to the association with the proteins. Consequently, dyes captured in the protein-SDS complexes remain highly fluorescent after destaining.

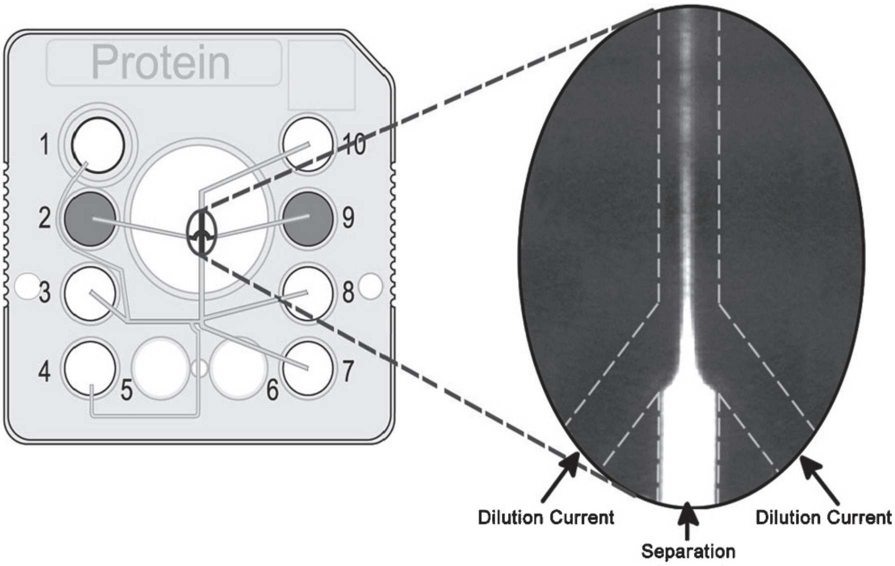


Fig. 1. Illustration of the destain intersection in a microfluidic chip for protein separation.

Fig. 2 illustrates the phenomenon in a plot of measured fluorescent intensity as a function of protein migration time when a detector is placed downstream of the destaining channel intersection. When the destain ratio, a ratio of flow from the destain channels to that from the separation channel, is high enough to break down the free micelles, the background signal drops significantly and the protein peak intensity goes up simultaneously, most likely due to the availability of more dyes for the bound micelles after they are released from the free micelles.

The time required for destaining of the free micelles is dictated by Brownian diffusion as described by the Einstein relationship:

$$t = x^2/2D$$

For free SDS micelles in the center streamline pinched laterally by two destaining solution streams as shown in Fig. 1, the diffusion distance into the SDS-free zone is about 15 μm, and the characteristic diffusion time is on the order of 200 ms. As a result, the location of the detector can be designed to be appropriately placed downstream of the destaining intersection to optimize the benefits of the on-chip operation. This destaining step in protein sizing is one good example of

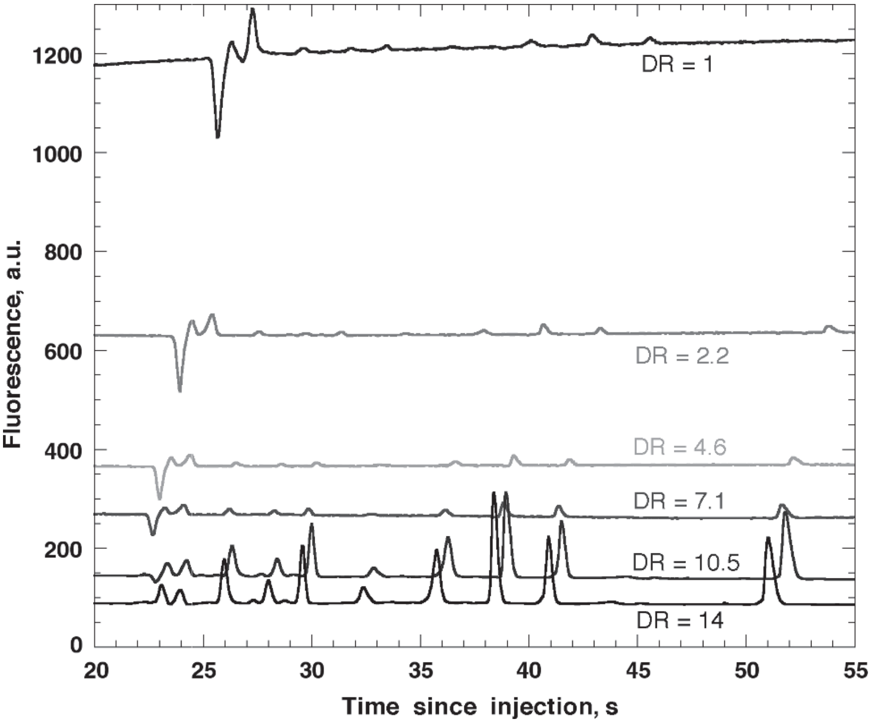


Fig. 2. A plot of fluorescence versus protein transit time at a range of destain ratios.

an integrated function easily performed on chip by exploiting the scaling laws of microfluidics that cannot be readily achieved in a conventional format.

2. Materials

Two types of microfluidic chips are available for protein sizing and relative quantitation. A planar chip format, where the samples are pipetted into designated well manually, is suitable for analyzing a low number of protein samples (up to 10). The planar chip format is used on the Agilent 2100 Bioanalyzer (http://www.agilent.com/) and Bio-Rad Experion Automated Electrophoresis System (http://www.biorad.com/). **Fig. 3** shows examples of these planar chips. The second type of chip format, a sipper chip, is designed for higher throughput analysis, tens to hundreds of samples at a time. The Caliper LabChip® 90 instrument (http://www.caliperls.com/) uses the sipper chip format. The sipper is a capillary attached to the microfluidic chip to enable automated introduction of protein samples from a

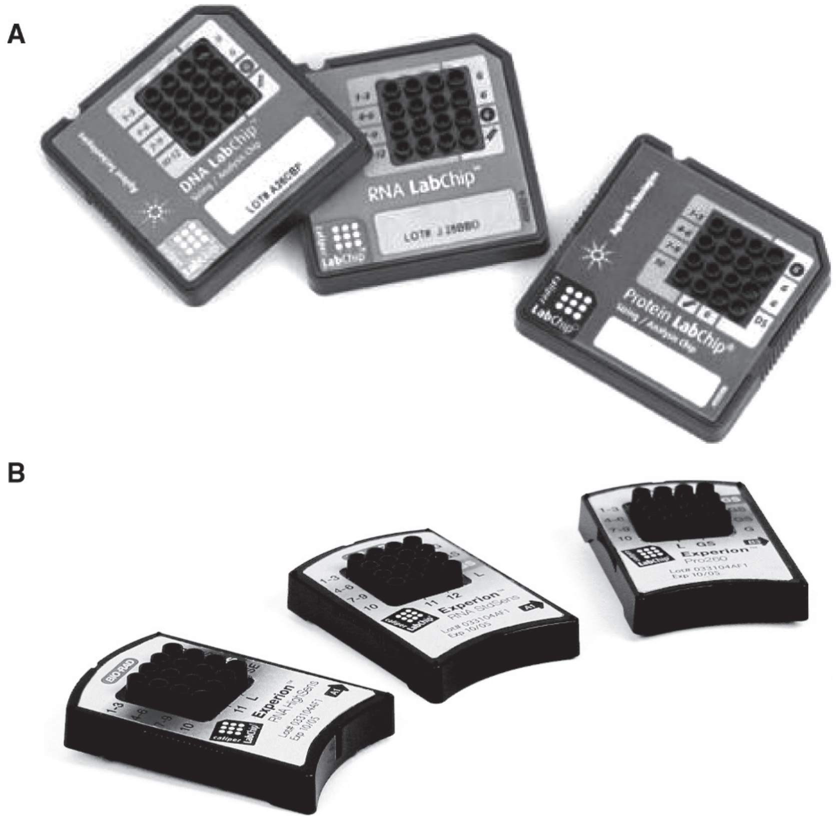


Fig. 3. Examples of planar LabChip® devices for separations. (**A**) Chips for the Agilent 2100 Bioanalyzer. (**B**) Chips for the Bio-Rad Experion.

standard 96 microtiter plate format onto the microchannel network of the chip. **Fig. 4** is a photograph of a sipper chip.

Fig. 5 illustrates the microchannel design layout of the planar chip for protein separation. The samples are manually pipetted into the reservoirs of the chip

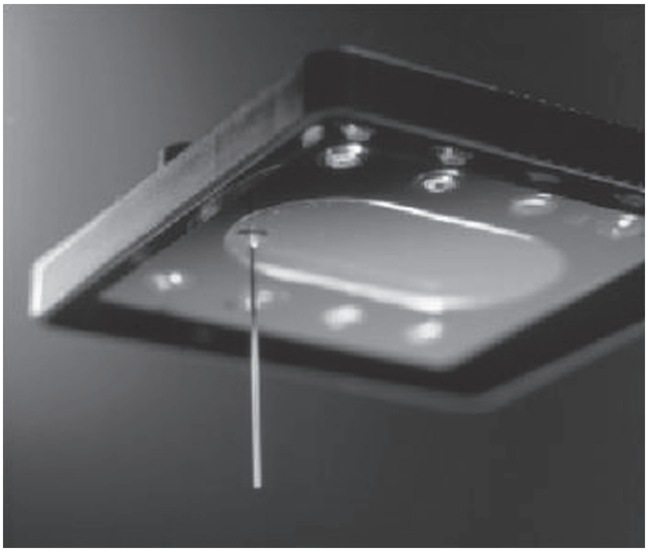


Fig. 4. A photograph of a sipper chip.

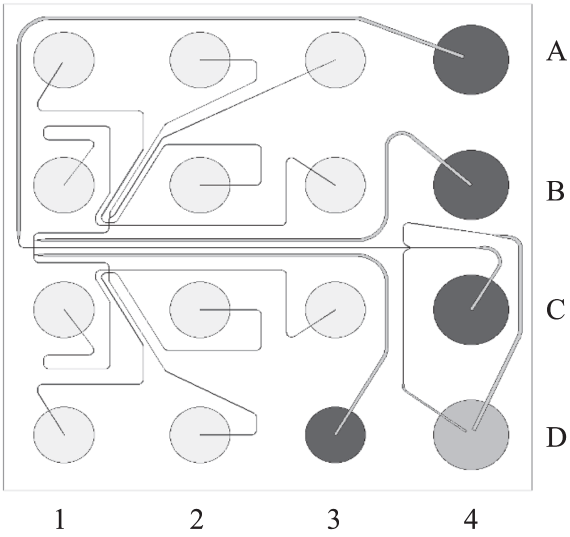


Fig. 5. The microchannel design layout of the planar protein chip.

after the microchannels are filled with the polymer sieving matrix. The matrix is a solution containing a high molecular weight polymer, poly(dimethyl methacrylate) or PDMA, in a Tris-Tricine containing buffer (the gel-matrix) mixed with a noncovalent fluorescent dye and SDS (the dye concentrate). Other reagents provided with the chips include a sample buffer, and a protein ladder with known molecular weight and concentration as a calibration standard for sizing and quantitation. The as-received reagents and buffers should be initially stored at −20°C. These reagents are supplied with 25 single-use, disposable chips in a kit, and should be stored at 4°C after opening and between use in order to avoid freeze-thaw cycles. The dye concentrate as well as the gel-dye mix should be kept in the dark due to light sensitivity of the dye. The details of the chip preparation method and the processes occurring inside the microchip are described in **Subheading 3.2.**

The microchannel design layout for the protein sipper chip is illustrated in **Fig. 6.** After the polymer solution with and without SDS-dye is properly introduced into the electrophoresis and destaining channels of the chip, the sipper dips into each of the microtiter well sequentially and samples a small quantity, about 100 nL, of protein sample, onto the chip by applying a small vacuum in the waste well. Subsequent injection and separation of samples are performed electrokinetically on chip. A single sipper chip can usually be used to analyze hundreds of samples. The details of the chip operations are provided in **Subheading 3.3**. Reagents provided with each LabChip® kit include a sample buffer, a denaturing buffer, a marker dye, a protein ladder, a destaining solution

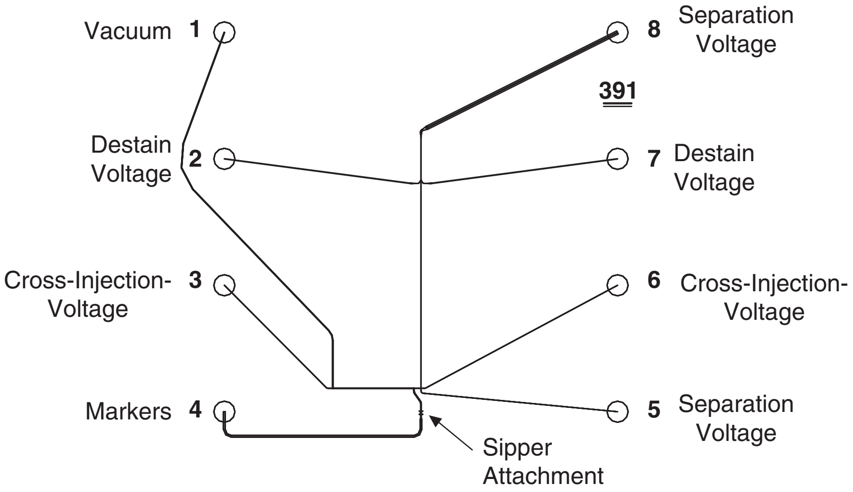


Fig. 6. The microchannel design layout of the protein sipper chip.

(PDMA polymer solution), and a dye concentrate (SDS mixed with an intercalating fluorescent dye). The dye concentrate and marker dye should be kept in the dark to avoid degradation. All reagents and buffers should be stored at 4°C after opening and between use. The as-received sipper chips should also be stored at 4°C and at room temperature after the first gel priming. Once the chip is primed, it can remain on the instrument for use throughout the day. Multiple batches of samples can be analyzed with the same chip without further reagent addition to the chip. After one 8-hr day use, the chip must be reprimed with fresh gel-dye mix prior to use the next day. Typically, each chip can be used for up to 300 samples if handled with good laboratory practices and care (*see* **Note 1**).

3. Methods

3.1. Denaturing Protein Samples

The protein samples and protein ladder are denatured in high concentration of SDS or LDS (lithium dodecyl sulphate), at >0.5%, either under reducing or non-reducing conditions. For reducing condition, β-mercaptoethanol or dithiothreitol is typically added to the denaturing solution. The following steps are recommended for denaturing protein samples:

1. The protein samples mixed with the denaturing solution according to the recommended protocol, are heat-denatured at 90–100°C for 3–5 min.
2. The samples are then further diluted with deionized water and/or sample buffer, usually by ~10–15 fold before use.

The protein samples should be analyzed in the same day after denaturization.

3.2. Planar Microfluidic Chips

The procedures to perform protein separations on planar chips consist of chip preparation, sample loading, and placing the chip onto the instrument. Chip preparation requires the complete filling of the separation channels of a dry chip with the polymer matrix mixed with SDS and dye (gel-dye mix) as follows:

1. First, 12 μL of the gel-dye mix is pipetted into the A4 well in the lower right corner of the well pattern of the chip (see **Fig. 5** for chip layout).
2. A priming station consisting of a syringe is used to fill the channels with the polymer solution by applying pressure of 3 atm at well A4 for 60 s.
3. Once the channels are filled, 12 μL of gel-dye mix is pipetted into wells B4, C4 and D3.
4. Next, 12 μL of the destaining solution (gel without SDS-dye) is pipetted into well D4.
5. Six μL each of sample diluted into the sample buffer containing lower and upper protein markers is pipetted in all 10 sample wells.
6. Six μL of the diluted ladder is pipetted into the ladder well D2.

The chip is now ready for loading onto the instrument.

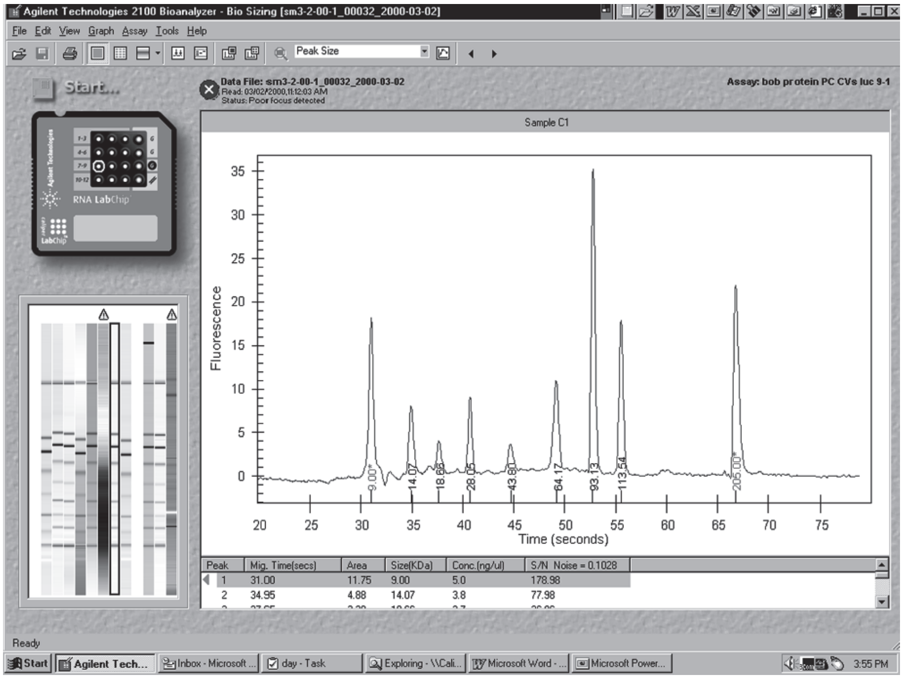
Inside the instrument, the chip rests on a heater block and there is an electrode block located on the lid that matches the well pattern on the chip. After the lid is closed, each electrode pin makes contact with the liquid in each of the 16 wells, and voltage and current can be applied to the channels through the reagent wells. The optics is located below the chip to excite and record the fluorescence of the intercalating dye. The sizing experiment commences by activating the start button on screen.

The following procedures have been programmed into the software script controlling the voltage and current, so they are transparent to the users. The script starts with a warm and focus step, which allows the chip to come to temperature equilibration with the heater block at 30°C and the auto focus function to locate and focus the light source onto the separation channel. During this time, the instrument also checks for electrical connections in all the wells before starting the separation analysis. The protein ladder is first loaded by applying an electric field between the source "ladder" well (D2) towards well B4. After the upper protein marker reaches the injection intersection, the voltage is switched well A4 to wells C4 and D4 for separation. A small aliquot of ladder protein with a band on the order of 50 μm is injected into the separation channel, and the protein species are separated by size through the SDS-dye containing polymer matrix followed by destaining and detection as described in Section 1. Overlap loading and separation of subsequent samples is performed to minimize the total analysis time. All the steps after the start of the experiment take about 30 min to complete. The results are digital recordings of fluorescence intensity as a function of transit time for the ladder and samples as shown in **Fig. 7.** The electropherograms can also be displayed as a virtual gel image by software, and a result table. The protein size is computed from the transit time as compared to a calibration curve determined by the transit time of the ladder after the upper and lower markers in the samples and ladder are aligned by the software. Relative protein concentration is computed from the area under each peak as compared to the known concentrations of protein in the ladder.

3.3. Sipper Microfluidic Chips

Unlike the planar chip, which is shipped dry, a sipper chip comes in a wet container in which the microchannels, the sipper, and the wells of the chip are filled with storage buffer. Before starting an experiment, the chip should be prepared using the following steps:

1. After the chip is removed from the container, all the wells are rinsed with filtered deionized water.
2. Contents in wells 5, 6, and 8 are replaced with 75 μL of polymer matrix containing SDS and dye, contents in wells 2 and 7 are replaced with 75 μL of polymer matrix


Agilent Technologies 2100 Bioanalyzer - Bio Sizing [sm3-2-00-1_00032_2000-03-02]
File Edit View Graph Assay Tools Help
Peak Size
Start...
RNA LabChip
Sample C1
Fluorescence
35
30
25
20
15
10
5
0
9.00*
14.07
18.66
28.05
43.80
64.17
93.13
113.54
205.00*
20
25
30
35
40
45
50
55
60
65
70
75
Time (seconds)
Ready
Start
Agilent Tech...
Inbox - Microsoft ...
day - Task
Exploring - \\Cali...
Microsoft Word - ...
Microsoft Power...
3:55 PM

Data File: sm3-2-00-1_00032_2000-03-02
Read: 03/02/2000,11:12:03 AM
Status: Poor focus detected

Assay: bob protein PC CVs luc 9-1

Peak	Mig. Time(secs)	Area	Size(KDa)	Conc.(ng/ul)	S/N Noise = 0.1028
1	31.00	11.75	9.00	5.0	178.98
2	34.95	4.88	14.07	3.8	77.98

Fig. 7. Data output of the 2100 Bioanalyzer protein run.

without SDS, and addition of 120 µL of wash buffer to well 3 (see **Fig. 6** for chip layout).

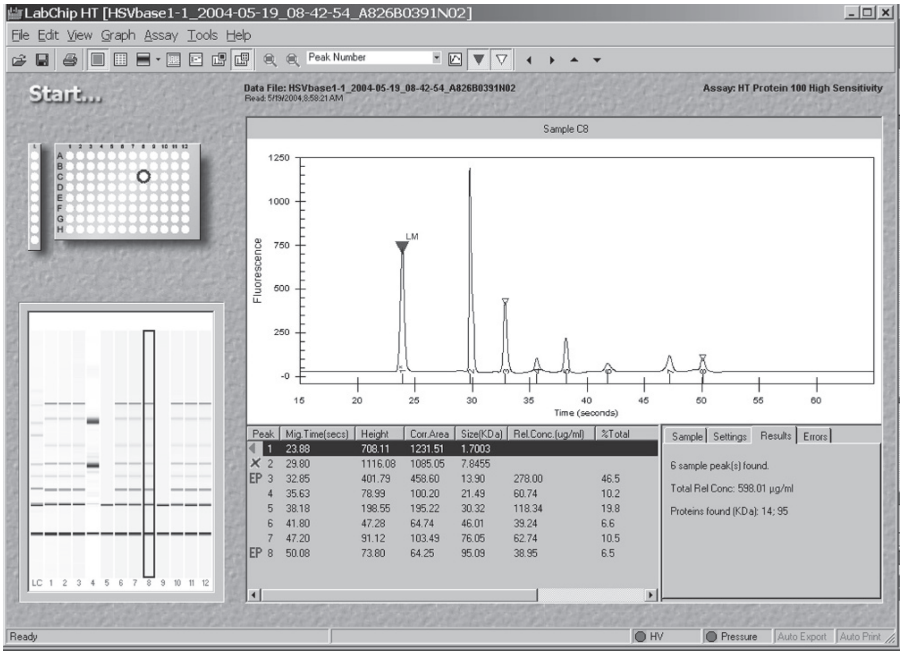
3. The chip is next placed in a priming station where the electrophoresis channels are filled with the polymer-dye matrix solution by applying a pressure of 3 atm to wells 2, 5, 6, 7, and 8 for 6 min.
4. The fluid in well 3 is then replaced with 75 µL of the polymer matrix.
5. The fluid in well 4 is replaced with 120 µL of marker dye solution.

The chip is now ready to be loaded in the instrument for high throughput analysis.

When the sipper chip is placed onto instrument at the bottom of the optics module, the sipper capillary hangs out from the enclosed module where it can interface with the microtiter plate placed on the plate holder. The plate holder sits on a XYZ robotic arm and can accommodate the microtiter plate containing denatured protein samples and two well strips, one for the ladder and one for sample buffer. The buffer well is used to rinse the capillary between samples to eliminate cross-contamination. Inside the instrument, the sipper chip rests on a heater block which maintains the chip at a constant temperature of 30°C throughout the experiment. The initial chip warm up and focus step consists of sipping sample buffer by applying a vacuum in the waste well followed by multiple injection and separation. The excitation laser is focused onto the separation channel using an autofocus mechanism.

At the start of an experiment, the sipper samples the ladder well to introduce onto the main channel about 100 nL of protein ladder by applying a small vacuum in the waste well. Once on chip, the protein sample from the sipper is mixed with the marker and flow toward the waste well 1. The mixture of protein and marker is next loaded onto the separation channel network electrokinetically by applying a voltage gradient between wells 3 and 6. Subsequently, the voltage is switched to wells 5 and 8 to inject a small aliquot of the mixture into the separation channel, which separates the protein species by size through the polymer matrix. Voltage is also applied to wells 2 and 7 for destaining as described in **Subheading 1.** The excitation laser and detector are located downstream of the destain intersection to record the fluorescence intensity of the protein fractions. During electrophoretic separation of the first sample, a second sample is simultaneously sipped from the plate to be ready for sample injection. The sipping operation of one sample does not affect the separation resolution of another due to the high matrix viscosity and shallower channel depth in the separation channel, which prevent significant pressure driven flow to occur in the separation channel. Such overlap sipping and separation processes are repeated sequentially until all the samples on the plate are analyzed. The protein ladder is sipped repeatedly after every 12 samples. A 96-well plate full of samples takes appropriately 1 hour and 20 min to analyze. The system can be used to analyze full or partially filled plates. It is also possible to do continuous or intermittent analysis throughout the day with a single chip that has been primed once.

The sizing of protein species in each sample is computed after the lower marker is aligned by software with that in the ladder sipped prior to the sample, and the transit times are compared with a calibration curve of ladder transit time versus known protein ladder size. The protein concentration for each peak is computed using the relative area under the peak as discussed before after the peak areas are normalized by the area of the marker dye peak. Alternatively, the protein concentration can be more accurately determined using an internal standard added to each sample. The separated proteins can be viewed as electrophoregrams and a virtual gel. The computed sizes, relative concentrations, and % purity for each protein within each well are presented in a table once two bracketing ladders per row have been analyzed (*see* **Fig. 8**). The software has an "expected protein" feature to mark protein peaks detected at a predetermined size range. Automated calculation of protein purity can also be enabled. The results from the tables can be exported for further postprocessing analysis.


File Edit View Graph Assay Tools Help
Peak Number
Start...
Sample C8
1250
1000
750
500
250
-0
Fluorescence
LM
15
20
25
30
35
40
45
50
55
60
Time (seconds)
A
B
C
D
E
F
G
H
LC 1 2 3 4 5 6 7 8 9 10 11 12
Sample
Settings
Results
Errors
Ready
HV
Pressure
Auto Export
Auto Print

LabChip HT [HSVbase1-1_2004-05-19_08-42-54_A826B0391N02]

Data File: HSVbase1-1_2004-05-19_08-42-54_A826B0391N02
Read: 5/19/2004,8:58:21 AM

Assay: HT Protein 100 High Sensitivity

	Peak	Mig.Time(secs)	Height	Corr.Area	Size(KDa)	Rel.Conc.(ug/ml)	%Total
◀	1	23.88	708.11	1231.51	1.7003		
✕	2	29.80	1116.08	1085.05	7.8455		
EP	3	32.85	401.79	458.60	13.90	278.00	46.5
	4	35.63	78.99	100.20	21.49	60.74	10.2
	5	38.18	198.55	195.22	30.32	118.34	19.8
	6	41.80	47.28	64.74	46.01	39.24	6.6
	7	47.20	91.12	103.49	76.05	62.74	10.5
EP	8	50.08	73.80	64.25	95.09	38.95	6.5

6 sample peak(s) found.

Total Rel Conc: 598.01 µg/ml

Proteins found (KDa): 14; 95

Fig. 8. Data output of a LabChip® 90 protein run.

4. Notes

1. In microfluidics chips, keeping bubbles and debris away from the channels is important for good device performance and lifetime. As in capillary electrophoresis, bubbles and debris in microchannels are usually detrimental to the separation performance and longevity of a microfluidic device. Before introducing any fluid onto the chip, it is highly recommended that it should be filtered with a micron- or submicron-pore filter first. During manual pipetting of solutions into chip wells, it is important to touch the tip of the pipette to the bottom of the small opening of the glass plate inside the well, not on top of the glass plate or to the side wall of the well, to avoid introducing or trapping bubbles in the well. Samples placed in tubes and microplates should be centrifuged in order to settle particulates to the bottom. Subsequent manual pipetting or automated sipping should be taken from the top half of the tube or microplate wells to minimize introducing particulates into the channels of the chip.

   The planar chips should always be run with all the sample wells filled with the recommended volume of fluid. If there are fewer than 12 samples to be used in one device, the remaining wells should be filled with an equivalent amount of buffer to balance the hydrodynamics. The chip will not run properly if any wells are left empty. It is extremely important that the buffer and sample in the wells are thoroughly mixed using the vortex mixer at the conditions specified before loading the chip onto the instrument, otherwise protein concentration determination will not be reproducible. The speed of the vortex mixer should be set to the indicated setting at 2400 RPM. To avoid cross contamination, the electrodes in the instrument should be cleaned periodically as recommended in the user manuals.

As mentioned in **Subheading 2,** the intercalating dye is light sensitive. Therefore, it is important to keep solutions containing the dye in the dark when not in use. Otherwise, when the dye degrades significantly, signal intensity and thus detection sensitivity could be affected.

Because new sipper chips and many reagents are refrigerated before use, adequate time should be allowed for the chip and reagents to reach room temperature before starting the chip preparation steps outlined in **Subheading 3.2.** If cold reagents are introduced into the chip, warming up of an aqueous solution to room temperature in an enclosed microchannel could cause outgassing especially under slight vacuum condition.

2. Thus far, the detection sensitivity of proteins in commercial microfluidic chips is about the same as Coomassie Blue to mid-range of Colloidal Coomassie. It is advisable not to decrease protein sample dilution from the recommended ratio to try to increase sensitivity. Because protein buffers vary a lot in composition and conductivity due to different protein processing conditions, decreasing denatured protein sample dilution from the recommended value would result in more ionic species associated with the protein sample being injected into the separation column. It has been found that certain components of protein buffers, such as some detergents, in relatively high concentration in the injected sample band can affect the baseline flatness of the electropherogram or the migration rate of the lower marker, potentially causing degradation in accuracy of sizing and relative concentration determination.
3. Microfluidic devices are now being more widely used for sizing and quantitation of proteins by research laboratories that previously had used SDS-PAGE and to a lesser degree SDS-CGE. With greater use of microfluidic platforms, one obvious question is how well the measured molecular weight and relative concentration compares between the different methods. The experimental data up to now show these approaches provide similar answers for most proteins. For several proteins, minor migration variation relative to other proteins that result in differences in the determined size has been reported. The differences appear to be due to thevariations in the staining efficiencies by the fluorescent dye. It has been accepted by the SDS-PAGE user community that migration rates and dye staining of

protein-SDS complex are somewhat dependent on the protein sequence and the specific reagents used. However, complete understanding of how these parameters depend on sequence and reagents does not yet exist.

Unlike double-stranded DNA, which can be described successfully by a worm-like chain model with a measured persistence length *(5),* there has been no satisfactory conformational description of a protein-SDS complex structure that has received wide acceptance. It was first thought that a protein-SDS complex adopts a short rod-like segments connected by flexible regions *(6).* The "necklace" model, describing the protein-SDS complex as consisting of a series of spherical micelles surrounded by protein segment and connected by protein strands *(7, 8),* is receiving increasing attention. With a lack of good understanding of protein-SDS conformation, however, it is difficult to ascertain the fundamental electrophoretic migration behavior. So far, phenomenological observation shows a linear relationship of electrophoretic mobility plotted against logarithmic molecular weight for protein sizing data generated by SDS-PAGE *(1).* Data obtained from the LabChip® 90 sipper devices also show such a relationship in the molecular weight range of 10 kD to 250 kD in a variety of PDMA polymer solutions as illustrated in **Fig. 9.** In contrast, an equivalent plot for dsDNA migration is not linear **(Fig. 10),** and the changes in slope represent changes in the DNA conformation during migration through the polymer matrix *(9).* Thus, it is

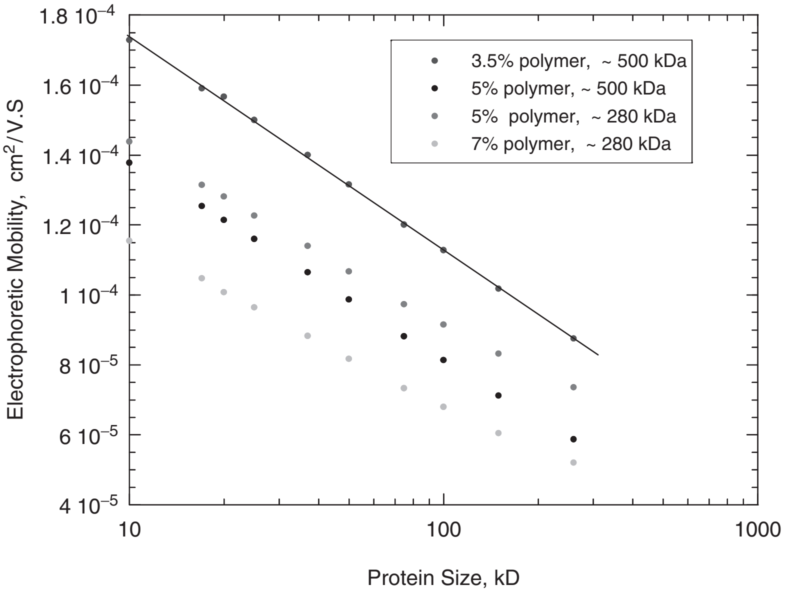


Fig. 9. A plot of electrophoretic mobility versus log protein size.

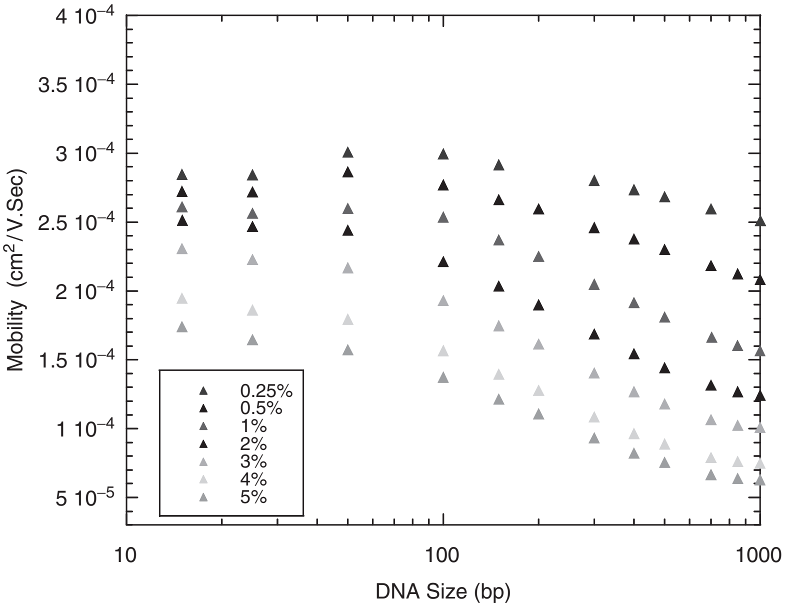


Fig. 10. A plot of electrophoretic mobility versus log DNA size.

reasonable to propose that protein-SDS complexes migrate very similarly in the conventional and microfluidic equivalent of SDS-PAGE for protein sizing. The small discrepancies observed in a gel image generated by a SDS-PAGE run versus the virtual gel image of a microfluidic sizing run are potentially due to the differences in the polymer matrix and dye used. Since the gel images of two SDS-PAGE runs generated by reagents made by two different manufacturers do not necessarily match, one should not expect a perfect match of separation resolution and relative staining intensities between a conventional gel image and one generated by a microfluidic chip on the same protein sample.

4. Analysis of whole cell lysates is essential for protein expression profiling studies. **Figure 11** shows a comparison of a lysate sample analyzed by a sipper chip and by SDS-PAGE. The over-expressed protein around 48 kD is detected well by bothmethods, although the data reproducibility appears better in the chip-based method. In analyzing lysates, it is important that the sample concentration to be within the upper limit of the recommended range, about 2000 ng/µL, in order to avoid the potential problem of sipper clogging by potential unsolubilized protein aggregates.

Development and production of antibody therapeutics require a significant amount of characterization and monitoring of stability and aggregation of these

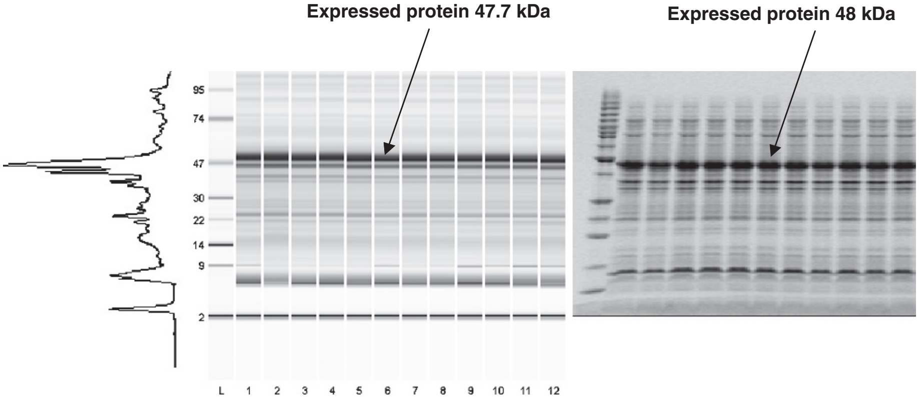


Fig. 11. A comparison of a LabChip® 90 virtual gel view and SDS-PAGE for a lysate sample (Data provided as the courtesy of Structural GenomiX of San Diego).

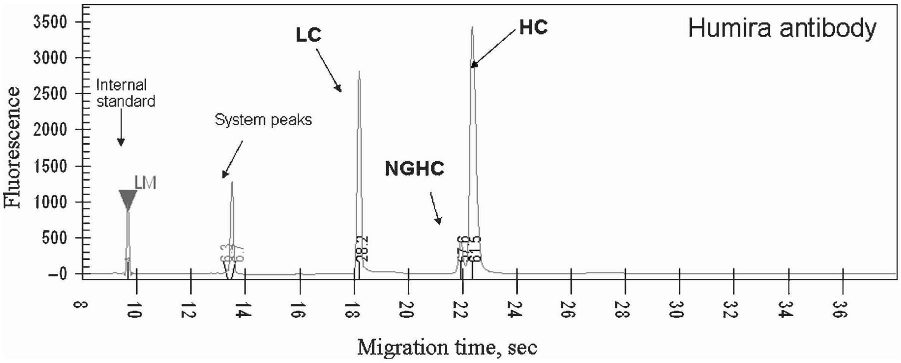


Fig. 12. Separation of reduced Humira (Abbott Laboratories) monoclonal antibody on Caliper LabChip 90. Abbreviations: light chain (LC), heavy chain (HC), and non-glycosylated heave chain (NGHC).

biological molecules. Microfluidic systems have been increasingly used for this application. **Figure 12** shows an electropherogram of separation of monoclonal antibody measured in the sipper chip system. The separation of light chain, heavy chain, and non-glycosylated heavy chain is comparable with data generated by SDS-CGE, but at a 60-fold reduction in separation time on a microchip ***(10).***

Another protein separation application demonstrated in chips is cereal protein characterization. **Fig. 13** shows an electrophoregram of wheat glutenin fractions. The composition of glutenin-subunit is an important predictor for the genetic potential of breeding lines, because these proteins are associated with differences in bread-making quality. Rapid separation and quantification of

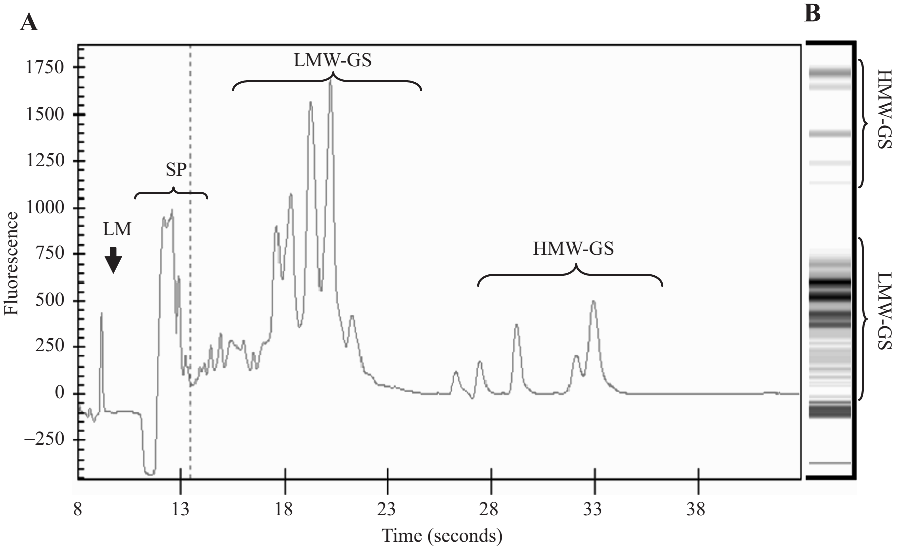


Fig. 13. (**A**) An electrophoregarm of wheat glutenin fraction under reduced conditions and (**B**) its corresponding virtual gel. Abbreviations: low molecular weight glutenin subunits (LMW-GS), high molecular weight glutenin subunits (HMW-GS), low marker (LM), and system peak (SP).

glutenin subunits have been achieved using microfluidic chip-based electrophoresis ***(11).*** However, the planar chips (Agilent 2100 Bioanalyzer platform) are limited to analysis of 10 samples at a time and thus not practical for large scale analysis. Glutenin subunits have also been analyzed on a high throughput integrated microfluidic-based platform (LabChip 90) ***(12).*** The electropherogram in **Figure 13** illustrates the separation of the HMW-GS and LMW-GS into two distinguished fractions with high resolution and fast analysis time.

It is well known in SDS-PAGE analysis that glycosylated proteins migrate slower than their expected molecular weights. Qualitatively similar phenomenon has been observed in microfluidic chip separation of glycosylated proteins. Therefore, similar precautions in data interpretation should be taken in chip-based analysis as in SDS-PAGE.

5. With some proper care and good laboratory practices, these microfluidics systems have been proven to be robust and labor-saving compared to the traditional SDS-PAGE. Perhaps more important, the output data is of high quality and available in a digital form which can be easily archived and shared with other laboratories across the company and around the world. Protein detection sensitivity down to silver-stain equivalence on microfluidic chips is not yet commercial, but approaches explored so far look promising ***(13)***. Moreover,

many research studies have also been reported on interfacing microfluidic chips with electrospray and MADLI mass spectrometry ***(14)*** and a polyimide LC/MS chip has recently been commercialized by Agilent. Microfluidics-based protein separation technologies have the potential to revolutionize the productivity of proteomics research in this era of increasingly more global community with lightning-speed demand for information sharing.

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Difference Gel Electrophoresis (DIGE)

David B. Friedman and Kathryn S. Lilley

1. Introduction

The proteome is a dynamic entity, constantly changing both in levels of protein expression as well as in posttranslational modification and localization, all of which are hidden in the static DNA code. The repertoire of techniques and associated instrumentation which is now being applied to proteomics experiments is expanding exponentially, and although a complete visualization of the proteome is still beyond reach of any single technique, several technology platforms exist that can provide complementary datasets. Proteomics in the clinical setting is also developing, providing a major impact on the way in which diseases will be diagnosed, treated and monitored ***(1).***

In particular, approaches involving two-dimensional (2-D) gel electrophoresis (2-DE) separations and subsequent protein identification using mass spectrometry (MS) have been useful for imaging thousands of intact proteins (including modified/proteolyzed forms) in a single run, but quantification has been challenging, particularly over a large sample set. Difference gel electrophoresis (DIGE) has proven to be a powerful quantitative technology for differential-expression proteomics on a global level, where the individual abundance changes for thousands of intact proteins can be simultaneously monitored in replicate samples over multiple variables with statistical confidence (*see* **Note 1**). This includes quantitative information on protein isoforms that arise due to a charged post translational modification that results in a change in the isoelectric point of the protein (such as acetylation or phosphorylation), and splice variants or protein processing events, all of which can be resolved for individual quantification and subsequent analysis by mass spectrometry.

DIGE is based on conventional 2-D gel technology that is capable of resolving several thousand intact proteins first by charge using isoelectric focusing (IEF)

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and then by apparent molecular mass using SDS-PAGE (2, 3) (*see* **Note 2**). Importantly, DIGE overcomes many of the limitations commonly associated with 2-D gels such as analytical (gel-to-gel) variation and limited dynamic range that can severly hamper a quantitative differential-expression study. This is accomplished using up to three spectrally resolvable fluorescent dyes (Cy2, Cy3 and Cy5, refered to as CyDyes) that enable low- to sub-nanogram sensitivities with $>10^4$ linear dynamic range, and then by multiplexing the prelabeled samples into the same analytical run (2-D gel). Multiplexing in this way allows for direct quantative measurements between the samples coresolved in the same gel, and is therefore beyond the limitations imposed by between-gel comparisons with conventional 2-D gels.

The highest statistical power of this multiplexing approach stems from the utilization of a pooled-sample internal standard comprised of an equal aliquot of every sample in the experiment (*see* **Subheadings 1.2.1., and 3.3.3.,**). With this method, two dyes (Cy3 and Cy5) are used to individually label two independent samples from a much larger experiment, and the Cy2 dye is used to label an internal standard which is comprised of an equal aliquot of proteins from every sample in the experiment. This pooled-sample internal standard is labeled only once in bulk to avoid additional technical variation, and enough is prepared and labeled to allow for an equal aliquot to be coresolved on each gel. The three differentially labeled samples are then coresolved on the same 2-D gel, after which direct measurements can be made for each resolved protein using the spectrally exclusive dye channels without interference from technical variation of the separation (gel-to-gel variation).

Rather than making direct quantitative measurements between the two samples in the gel, the measurements are instead made relative to the Cy2 signal from the internal standard for each resolved protein. This is done for each protein form under investigation separately, and so each feature is quantified relative to its own unique internal standard. The Cy2 signal should be the same for a given protein across different gels because it came from the same bulk mixture/labeling; therefore, any difference represents gel-to-gel variation, which can be effectively neutralized by normalizing all Cy2 values for a given protein across all gels. Using the Cy2 signal to normalize ratios between gels then allows for the Cy3:Cy2 and Cy5:Cy2 ratios for each protein within each gel to be normalized to the cognate ratios from the other gels, encompassing all samples. Each gel may contain different (and/or replicate) samples in the Cy3 and Cy5 channels, but all can be quantified relative to each other because each is measured to the cognate Cy2 signal from the internal standard present on each gel. With the use of sufficient replicates, a plethora of advanced statisitcal tests can be applied to highlight proteins of interest whose change in expression are related to the biology under investigation. *Since the technical noise is low, these vital replicates should be independent (biological) replicates.*

In a final step, specific proteins of interest are then identified using standard mass spectrometry approaches on gel-resolved proteins that have been excised and proteolyzed into a discrete set of peptides. Briefly, excised proteins are subjected to in-gel digestion with trypsin protease (typically), and mass spectrometry (MS) is used to acquire accurate mass determinations on the resulting peptides and peptide fragments generated using tandem mass spectrometric approaches. The mass spectral data are then used to identify statistically significant candidate protein matches through sophisticated computer search algorithms that compare the observed MS data with theoretical peptide masses (using data generated by peptide mass fingerprinting) or collision-induced fragmentation patterns (obtained from tandem mass spectrometry) generated *in silico* from protein sequences present in databases.

1.1. Optimizing Sensitivity and Resolution

There are currently two forms of CyDye labelling chemistries available: minimal labeling involving the use of N-hydroxy succinimidyl ester reagents for low-stoichiometry labeling of proteins largely via lysine residues, and saturation labeling which utilize maleimide reagents for the stoichiometric labeling of cysteine sulfhydyls.

The most established DIGE chemistry is the "minimal labeling" method, which has been commercially available since July 2002. Here the CyDye DIGE Fluors are supplied as *N*-hydroxy succinimidyl (NHS) esters, which react with the ε-amine groups of lysine side-chains. The three fluors are mass matched (c. 500 Da), and carry an intrinsic +1 charge to compensate for the loss of each proton-accepting site that becomes labeled (thereby maintaining the pI of the labeled protein). Each dye molecule also adds a hydrophobic component to proteins, which along with MW, influences how proteins migrate in SDS-PAGE.

Minimal labeling reactions are optimized such that only 2–5% of the total number of lysine residues are labeled, such that on average a given labeled protein would contain only one dye molecule. This is necessary because lysine is very prevalent in protein sequences, and multiple labeling events may affect the hydrophobicity of some proteins such that they may no longer remain soluble under 2-DE conditions. Although a given protein form may exhibit specific labeling efficiencies, these will be the same for labeling with all three dyes, allowing for direct relative quantification. Minimal labeling with CyDye DIGE fluors is very sensitive, comparable to silver-staining or many post electrophoretic fluorescent stains (*ca.* 1 ng, *see* **Subheading 2.2.6**), but with a linear response in protein concentration over four orders of magnitude ***(4)*** (*see* **Note 3**).

For maleimide-labeling of the cysteine sulfhydryls, the overall lower cysteine content in proteins allows for labeling of these residues to saturation without increasing the overall hydrophobicity of the proteins to cause insolubility problems. Saturation labeling is ultimately more sensitive (150–500 picograms, and even more so for proteins with high cysteine content). Its use is not as commonplace, most likely due to the availability of only Cy3 and Cy5 with this chemistry (*see* **Note 4**), the fact that it is blind to the small but significant population of non-cysteine containing proteins, and the additional optimization of complete cysteine reduction necessary for reproducible labeling. For these reasons, saturation DIGE is usually reserved for experiments where samples are limited, where the advantage of the increased sensitivity out-weigh these additional considerations.

To maximize the information that can be gained from experiments which utilize the DIGE technology, it is imperative that resolution of protein species within gels is optimized. Although single 2-DE runs can resolve proteins with pI ranges between pH 3–11 and apparent molecular mass ranges between 10–200 kDa, higher resolution and sensitivity can be obtained by running a series of medium range (e.g. pH 4–7, 7–11) and narrow range (e.g., pH 5–6) isoelectric focusing gradients with increasing protein loads, leading to an overall more comprehensive proteomic analysis ***(2, 3, 5)*** (*see* **Note 5**). This is analogous to gaining increased resolution and sensitivity in an LC/MS-based strategy by using multiple HPLC columns with different affinity chemistries (e.g., MudPIT ***(6)***). Much of the sensitivity limitation associated with 2-D gels can be attributed to the analysis of unfractionated, whole-cell and whole-tissue extracts. Additional sensitivity can be gained via enrichment for the proteins of interest, such as by analyzing prefractionated or subcellular samples, or immune complexes. However, the additional experimental manipulations required for prefractionation introduce more technical variation into the samples and necessitates increased independent (biological) replicates (which of course can be accommodated with the DIGE internal standard methodology).

The identification of proteins of interest using mass spectrometry can be performed directly from the DIGE gels when protein amounts have been optimized in this way (*see* **Subheading 3.5.,**). Alternatively, some experimental approaches perform the DIGE analysis using "analytical" gels with lower protein amounts, followed by protein excision from a secondary, "preparative" gel with higher protein amounts. This approach has its advantages when dealing with small sample amounts, such is often the case using the Saturation dye chemistries, but is also prone to uncertainties that arise due to the disproportionate amount of protein loading (*see* **Note 6**). The methods presented in this protocol are for optimization of both the DIGE data as well as material for subsequent mass spectrometry using high protein loads.

1.2. Optimizing Statistical Significance

1.2.1. Using the Internal Standard

The ability to coresolve and compare two or three samples in a single gel is attractive because it allows for direct relative quantification for a given protein without any interference from gel-to-gel variations in migration and resolution, removing the need for running replicate gels for each sample (similar to stable isotope LC/MS-based strategies) ***(44–47)***. This approach has limited statistical power, however, since confidence intervals are determined based on the overall variation within a population (*see* **Subheading 3.6.2.,**).

Many researchers new to DIGE technology are not immediately aware of the increased statistical advantage and multiplexing capabilities of DIGE when combining this approach with a pooled-sample mixture as an internal standard for a series of coordinated DIGE gels ***(7).*** This design will allow for repetitive measurements (vital to any type of experimental investigation), and in such a way as to control both for gel-to-gel variation and provide increased statistical confidence. Importantly, these replicates should be from independent experiments (biological replicates) rather than multiple samples from the same experiment (technical replicates) (*see* **Subheading 1.2.2.,**). In this way, statistical confidence can be measured for each individual protein based on the variance between multiple measurements, independent of the variation in the population. Incorporating independently prepared replicate samples into the experimental design also controls for unexpected variation introduced into the samples during sample preparation.

This more complex and statistically powerful experimental design is accomplished by using one of the three dyes (usually Cy2) to label an internal standard which is comprised from equal aliquots of protein from all of the samples in an experiment. The total amount of the Cy2-labeled internal standard is such that an equal aliquot can be coresolved within each DIGE gel that also contains an individual Cy3- and Cy5-labeled sample from the experiment. Since this standard is composed of all of the samples in a coordinated experiment, each protein in a given sample should be represented in the mixture and thus serve as an unique internal standard for that protein (*see* **Note 7**). Direct quantitative comparisons are made individually for each resolved protein between the Cy3- or Cy5-labeled samples and the cognate protein signal from the Cy2-labeled standard for that gel (without interference from gel-to-gel variation), enabling the calculation of standardized abundance for every spot matched across all gels within a multigel experiment. The individual signals from the internal standard are also used to normalize and compare between each in-gel direct quantitative comparison for that particular protein from the other gels. Using the Cy2-labeled standard in this fashion, therefore, allows for more precise and complex quantitative comparisons between gels, including independent (biological) sample repetition (**Fig. 1**).

Importantly, the internal standard experimental design allows for the identification of significant changes that would not have been identified if the analyses were performed separately, even when using Cy3- and Cy5-labeled samples on the same DIGE gel ***(8).*** This experimental design also allows for multivariable analyses to be performed in one coordinated experiment, whereby statistically

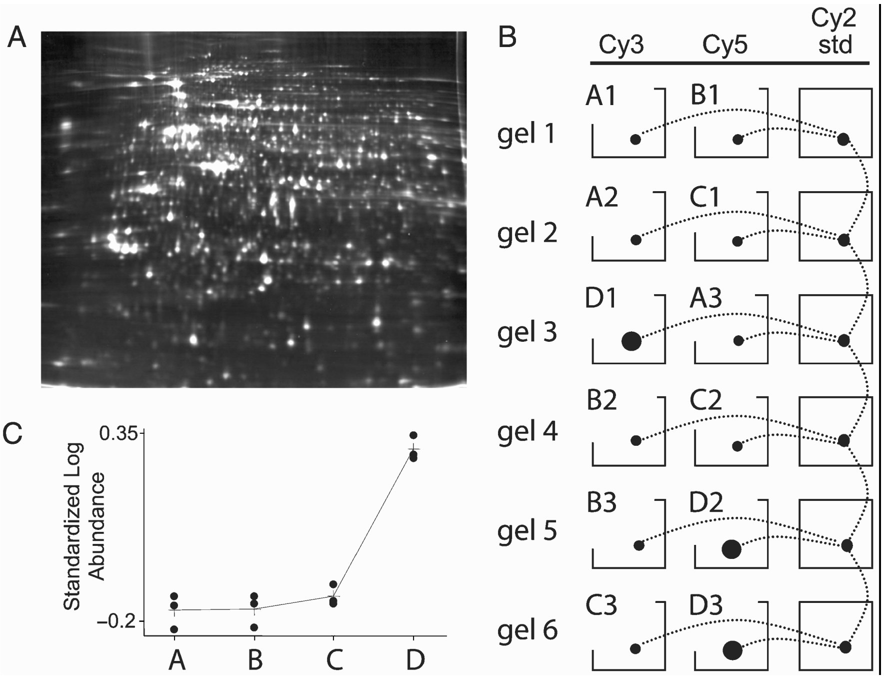

Fig. 1. Illustration of DIGE and experimental design using the mixed-sample internal standard. **(A)** Representative gel from a 6-gel set containing three differentially labeled samples: Cy2-labeled internal standard, Cy3-labeled sample #1, and Cy5-labeled sample #2. The individual protein forms all coresolve in this one gel, but these three independently labeled populations of proteins can be individually imaged using mutually exclusive exitation/emission properties of the CyDyes. **(B)** Schematic of the sample loading matrix indicating gel number, Cydye labeling and three replicates (indicated as "1, 2, and 3") of the four conditions being tested (A, B, C, and D). Within the boxed regions representing each labeled sample is depicted a theoretical protein that is upregulated in condition D. Dotted lines illustrate how the protein signals from each sample are directly quantified relative to the Cy2 internal standard signal for that protein without interference from gel-to-gel variation, and how the Cy3:Cy2 and Cy5:Cy2 intra-gel ratios are normalized between the 6 gels. **(C)** A graphical representation of the normalized abundance ratios for this theoretical protein change. Adapted from ***(5).***

significant abundance changes can be quantitatively measured simultaneously between several sample types (e.g., different genotypes, drug treatments, disease states), with repetition and without the necessity for every pair-wise comparison to be made within a single DIGE gel ***(9, 10)*** (*see* **Note 8**).

1.2.2. Assessing Intersample Variation

Even under the best-controlled situations there is always room for technical variation in sample preparation. Moreover, there will always be some degree of biological "noise" that represents fluctuations in protein expression that fall into the normal range. In the majority of cases, however, biological variation usually outweighs technical variation. Clinical proteomic experiments are especially hampered by biological noise that describes the variation between patient samples that is not associated with disease. The largest proportion of this variation comes from biological diversity, but a significant amount may also come from variable collection and storage of biological samples. It is of vital importance to identify changes in protein expression that describe the biological processes under investigation rather than variation derived from technical or biological noise.

In order to gain the more robust data sets necessary to be able to draw accurate conclusions, it is therefore of paramount importance to collect and store samples using very stringent and closely adhered to protocols. For clinically related samples it is also necessary to assess the biological variation within the population being tested and also within a single individual. Interindividual variation has been the focus of several studies ***(11, 12)*** and determining a typical diversity within a single patient (i.e. taking longitudinal samples and assessing variability in protein abundance) and between patients will determine the minimum number of patient samples required for an experiment. This is an essential step before embarking on any large-scale and potentially costly DIGE experiment. Without this type of pretest, the results of underpowered experiments run the risk of being peppered with false information (both false positives and negatives).

As with all complex technologies, the DIGE technique itself is subject to technical variation which will be laboratory specific to a greater or lesser extent. However, the amplitude of this variation is generally outweighed by the biological variation associated with a typical sample set ***(13).***

1.2.3. Univariate Statistical Analyses

To date, the majority of published quantitative proteomics studies using the DIGE technology have applied a univariate test, such as a Student's t-test or Analysis of Variance (ANOVA), to identify protein species with significant changes in expression ***(14).*** These tests calculate the probability (p) that the samples being compared are the same and therefore any apparent change in expression occurs by chance alone. An expression change is considered significant if the calculated p-value falls below a prescribed significance threshold, typically 0.05 (whereby 1 in 20 tests may give a change in expression by chance). For more stringent analyses, a p-value of 0.01 is often used as the significance threshold.

When employing these tests on DIGE datasets there are several factors that must be considered if correct assumptions are to be made from ensuing analyses. Student's t-tests and ANOVA assume that the data achieved is normally distributed and that any variance is homogeneous. The measurement and correction of systematic bias within DIGE experiments has been the subject of several studies which chart methods to optimize normalization of data sets ***(15–17).***

Another important consideration is that of false discovery rate (FDR) which could arise as a result of statistical tests such as the ones described above. These tests involve the simultaneous and independent testing of thousands of protein features. The probability of a false-positive being recorded for each test is such that a substantial number of false positives may accumulate. There are several approaches to determine the FDR and adjust *p*-values to compensate for this, the most widely used to date being the Benjamini and Hochberg method, whose use in conjunction with DIGE data has been described by Fodor et al. ***(15).***

1.2.4. Multivariate Statistical Analyses

Discovery phase proteomics often produce large lists of proteins that are identified as changing significantly in the experiment, many of which may well be false-positives. Another complementary approach to overcome issues surrounding univariate tests is the application of additional multivariate statistical analyses to these datasets, which can help to filter out false-positives that result from whole-sample outliers (i.e., sample mis-classification and/or poor sample preparation technique). These analyses, such as Principal Component Analysis (PCA), partial least squares discriminate analysis (PLS-DA) and unsupervised hierarchical clustering (HC) (*see* **Figs. 2** and **3**) have recently been applied to DIGE datasets ***(5, 18–26).*** Raw and normalized data can be exported from most DIGE software solutions (e.g., DeCyder, Progenesis), and several multivariate analyses are now part of an extended data analysis software module as part of the DeCyder suite of software tools (GE Healthcare) which was specifically developed for DIGE analysis (*see* **Subheading 3.6.,**). Similar capabilities can also be found in newer versions of the Progenesis software (Non-Linear Dynamics) and Delta2D [Decodon].

These multivariate analyses work essentially by comparing the expression patterns of all (or a subset of) proteins across all samples, using the variation of expression patterns to group or cluster individual samples. Technical noise (poor sample prep, run-to-run variation) and biological noise (normal differences between samples, especially present in clinical samples) is almost always associated with any analytical dataset of this nature, and may well override any variation that arises due to actual differences related to the biological questions being tested. Unsupervised clustering of related samples, therefore, adds additional confidence that a "list of proteins" changing in a DIGE experiment are not arising stochastically ***(5).***

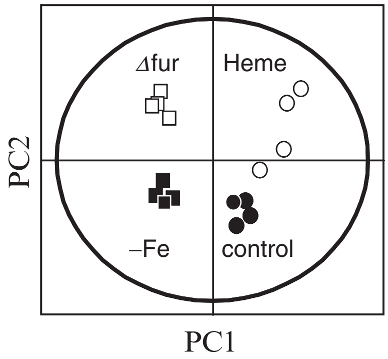

Fig. 2. Illustration of the use of Principal Component Analysis. DIGE was used to analyze changes in Staphylococcus proteins in reponse to genetic and chemical alterations affecting iron utilization. Adapted from *(18)*.

1.3. DIGE in the Clinical Setting

Although the potential for DIGE to address clinical studies is only beginning to be addressed (for example, see ***(23, 24)***), many studies have been published demonstrating the feasibility and benefit of DIGE/MS using small patient cohorts for preliminary studies in colon ***(8),*** liver ***(27–29),*** breast ***(30, 31),*** esophageal ***(32, 33),*** and pancreatic cancers ***(34),*** as well as other important clinical studies such as SARS ***(35).*** Many studies also explore the important benefit of procuring samples using laser capture microdissection (LCM) for a highly enriched population of the cells under study ***(10, 24, 36–38).*** These LCM studies necessitate the use of the saturation chemistry owing to the increased sensitivity but limited multiplexing power, and typically require secondary preparative gels with higher protein loads to enable protein identification by mass spectrometry.

The study of Suehara et al. ***(23)*** represents the utility of a multivariable DIGE/MS analysis with an extended sample set pertinent for a clinical study. Eighty soft-tissue sarcoma samples comprising seven different histological backgrounds were analyzed. Using the saturation DIGE fluors, individual samples were labeled with Cy5 and multiplexed with a pooled-sample internal standard (labeled in bulk with Cy3) for each DIGE gel. Using high-resolution 2-D gel separations and a combination of multivariate statistical tools (support vector machines, leave-one-out cross-validation, PCA and HC), these studies identified a small subset of proteins including tropomyosin and HSP27 that were able to discriminate between the different classes of tumors. HSP27 in particular was part of a subclass of discriminating proteins that could distinguish between leiomyosarcoma and malignant fibrous histiocytoma (MFH), as well as correlate with patient survival between low-risk and high-risk groups. HSP27 has long been associated with prognosis in MFH as well as in other human carcinomas ***(39).***

This chapter assumes a solid understanding in 2-D gel electrophoresis, and will focus on the design and implementation of the DIGE method using the pooled-sample internal standard methodology and the minimal dye chemistry for Cy2, Cy3 and Cy5, with notes provided for saturation labeling chemistry.

2. Materials

2.1. Cell lysis buffers

1. TNE: 50 m*M* Tris-HCl pH 7.6, 150 m*M* NaCl, 2 m*M* EDTA pH 8.0, 2 m*M* DTT, 1% (v/v) NP-40.
2. RIPA buffer: 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS.
3. 2-D gel electrophoresis lysis buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 2 mg/mL DTT, 50 mM Tris-HCl pH 8.0.
4. ASB14 lysis buffer: 7 *M* urea, 2 *M* thiourea, 2% amidosulfobetaine 14, 50 m*M* Tris-HCl pH 8.0.

Note: Depending on the sample, it may also be necessary to add protease inhibitors (Complete protease inhibitor cocktail tablets. Roche Diagnostics) and phosphatase inhibitors (sodium pyrophosphate (1 m*M*), sodium orthovanadate (1 m*M*), beta-glycerophosphate (10 m*M*) and sodium fluoride (50 m*M*)) to the chosen lysis buffer (*see* **Subheading 3.1**).

2.2. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

1. Immobilized pH gradient strips and accompanying ampholyte mixures can be purchased from a number of commercial vendors. Strip lengths vary from 7 cm to high-resolution 24 cm strips, and pH ranges vary from wide-range (e.g. pH 3–11) to high-resolution narrow-range (e.g. pH 5–6) strips.
2. Bind silane working solution (50 mL): 40 mL ethanol, 1 mL acetic acid, 50 μL bind silane solution (GE Healthcare), 9 mL water (*see* **Note 9**).
3. 4x separating gel buffer: 1.5 M Tris-HCl pH 8.8.
4. 30% acrylamide:bis-acrylamide (37.5:1), N,N,N,N -Tetramethyl-ethylenediamine (TEMED), and ammonium persulfate.
5. 10x SDS-PAGE running buffer (1 L): 30.25 g Tris-base, 144.13 g glycine, 10 g SDS (0.1%).
6. Fixing solution for SyproRuby staining (1 L): 100 mL methanol, 70 mL acetic acid, 830 mL water. SyproRuby stain is available form several commercial sources and can be substituted by other fluorescent total-protein stains such as Deep Purple (GE Healthcare), LavaPurple (Fluorotechnics), or Flamingo Pink (BioRad).
7. 2nd dimensional equilibration buffer: 6 M urea, 50 mM Tris-HCl pH8.8, 30% glycerol, 2% SDS, trace bromophenol blue.

8. Water-saturated butanol (*see* **Note 10**).
9. Dithiolthreitol (store dessicated).
10. Iodoacetamide (store dessicated, keep in the dark).

2.3. DIGE Labeling

1. N,N-dimethyl formamide (DMF) (*see* **Note 11**).
2. Labeling (L) buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-base (do not pH, but ensure that pH of final solution is between 8.0–9.0), 5 mM magnesium acetate (*see* **Note 12**). Alternatively, 4% CHAPS can be replaced with 2% ASB14, especially in cases where membrane rich samples are being utilized.
3. Rehydration (R) buffer: 7 M urea, 2 M thiourea, 4% CHAPS, 2 mg/mL DTT (13 mM; 2 %).
4. Cyanine dyes with NHS-ester chemistry for minimal labeling (Cy2, Cy3, Cy5), and with maleimide chemistry for saturation labeling (Cy3 and Cy5) are available from GE Healthcare as dry solids.
5. Quenching solution (for minimal labeling): 10 mM lysine.
6. Dithiothreitol reduction stock solution: 200 mg/mL DTT.

3. Methods

DIGE is a powerful technique for quantitative multivariable differential-expression proteomics. However, the quality of the data will only be as good as the quality of the underlying 2-D gel electrophoresis technology upon which it is based. The main focus of this chapter is to provide detailed notes on the DIGE technology, however, some key considerations to successful high-resolution 2-D gel electrophoresis are also provided. This section describes methods associated with labeling using minimal CyDyes.

3.1. Sample Preparation

The key to success for any analytical measurement begins with robust sample preparation. This not only includes the buffers and materials used, but also the nature of the samples and the way in which they are procured. The addition of exogenous materials (such as DNAse, RNAse), or allowing for uncontrolled manipulation of the sample (such as conditions that may lead to proteolysis) can severely hamper and sometimes completely prevent an analysis. Care should be taken to ensure against common laboratory contaminants (e.g., mycoplasma for tissue culture) that if present may be detected as significant changes using DIGE, either due to the presence in a subset of samples, or by responding to the experimental perturbation.

1. Prepare protein extracts using any method of preference. The appropriate amount of protein can be subsequently precipitated prior to resuspension in the CyDye labeling buffer (*see* **Subheading 3.2**). Ensure against proteolysis and loss of posttranslational modifications (e.g., phosphorylation) as this is of monumental importance.

Care should be taken not to use reagents that will resolve on the 2-D gel, such as soybean trypsin inhibitor. Small molecule inhibitors such as aprotinin, leupeptin, pepstatinA, antipain, AEBSF, sodium orthovanadate, okadaic acid and microcystin, among others, are far better choices.

2. Lyse cells using standard lysis buffers such as TNE, RIPA buffers, or even the buffers used for 2-D gel electrophoresis. All of these buffers have the capability of producing high-resolution samples for 2-DE. In most cases, the presence of reagents that would otherwise interfere with CyDye labeling (such as those that contain primary amines) will be removed prior to labeling by protein precipitation (*see* **Subheading 3.2**).
3. Sonicate cells if necessary to improve sample quality. Sonication improves sample quality by disrupting nucleic acids which are subsequently removed by sample cleanup (*see* **Subheading 3.2**) along with phospholipids. Both of these non-proteinaceous ionic components can obliterate the resolution during isoelectric focusing.
   Short bursts with a tip-sonicator are suggested. It is important to keep the system chilled, especially in the presence of urea-containing samples that should never be heated (*see* **Note 12**).
4. Determine the protein concentration of the sample using a system that is compatible for the buffer that the proteins are extracted in. CHAPS and thiourea in the buffers used for DIGE, although adequately chaotropic, interfere with either the Bradford or BCA assays, making the data inaccurate and unreliable. In these cases, aliquots should be precipitated prior to quantification in a suitable buffer, or the use of a detergent compatible assay should be utilized.
5. Aim to use a protein concentration between 1–10 mg/mL. Too dilute and it will be difficult to quantitatively recover proteins following precipitation clean-up (*see* **Subheading 3.2**); too concentrated and it will be difficult to accurately dispense the appropriate volume for the experiment. Freeze/thawing should also be kept to a minimum; freezing samples in 1 mL aliquots or less will usually suffice.

3.2. Sample Clean Up.

The desired amount of sample to be used in the experiment should be precipitated prior to labeling. This both removes non-proteinaceous ions from the sample (e.g., nucleic acids, phospholipids) that can interfere with IEF, and also transfers the proteins into a labeling buffer optimized for CyDye labeling and subsequent IEF. Determine how much total protein will be on each gel, and precipitate ½ of that amount for each sample to be run on that gel. This is straightforward for a two-component separation, but also works out for the multigel experiments where 1/3 of the total protein amount on each gel comes from the pooled-sample internal standard (*see* **Table 1**). Precipitate only what is needed for each sample for the experiment; too much material may create pellets that are difficult to resolubilize completely.

Many precipitation methods are available, the following is a MeOH/$CHCl_3$ protocol that works well for DIGE, and can be easily performed in 1.5 mL tubes (adapted from ***(40)***):

Table 1
Experimental Design for CyDye Labeling Using a Pooled-Sample Internal Standard

	Samples						
	Gel 1		Gel 2		Gel 3		
	Control-1	Treated-1	Control-2	Treated-2	Control-3	Treated-3	pool
Precipitated amount	150 µg	150 µg	150 µg	150 µg	150 µg	150 µg	
L-buffer	24 µL	24 µL	24 µL	24 µL	24 µL	24 µL	
Aliquot	16 µL	16 µL	16 µL	16 µL	16 µL	16 µL	8 µL (x6)
Cy2							6 µL
Cy3	2 µL		2 µL			2 µL	
Cy5		2 µL		2 µL	2 µL		
	30 min on ice in the dark						
Lysine (quench)	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	6 µL
	10 min on ice in the dark						
Total vol	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	60 µL
	For each gel, combine the quenched Cy3-and Cy5-labeled samples and add 1/3 of the quenched Cy2-labeled pooled mixture						
	20 + 20 + 20 µL		20 + 20 + 20 µL		20 + 20 + 20 µL		
2x R-buffer	60 µL		60 µL		60 µL		
total	120 µL		120 µL		120 µL		
R-buffer	to V_f		to V_f		to V_f		

This table illustrates a typical DIGE labeling experiment, as described in **Subheadings 3.2** and **3.3.**

1. Bring up predetermined amount of protein extract to 100 µL with water.
2. Add 300 µL (3-volumes) water.
3. Add 400 µL (4-volumes) methanol
4. Add 100 µL (1 volume) chloroform
5. Vortex vigorously and centrifuge; the protein precipitate should appear at the interface.
6. Remove the water/MeOH mix on top of the interface, being careful not to disturb the interface. Often the precipitated proteins do not make a visibly white interface, and care should be taken not to disturb the interface.
7. Add another 400 µL methanol to wash the precipitate.
8. Vortex vigorously and centrifuge; the protein precipitate should now pellet to the bottom of the tube.
9. Remove the supernatant and briefly dry the pellets in a vacuum centrifuge.
10. Resuspend the pellets in a suitable amount of CyDye labeling buffer (L-buffer, see **Table 1**).

An alternative widely used precipitation method is as follows:

1. Add 5 volumes of cold 0.1 *M* Ammonium acetate in methanol.
2. Leave at −20°C for 12 hr or overnight
3. Centrifuge at ~3000 rpm (1400 × g) for 10 min at 4°C and remove the supernatant.
4. A pellet of protein should be visible at this stage
5. To wash the pellet, add 80% 0.1 *M* ammonium acetate in methanol and mix to resuspend the protein.
6. Centrifuge at 3000 rpm (1400 × g) for 10 min at 4°C and remove the supernatant.
7. To dehydrate the pellet add 80% acetone and resuspend the pellet by mixing.
8. Centrifuge at 3000 rpm (1400 × g) for 10 min at 4°C and remove the supernatant.
9. Dry pellet for 15 min by leaving open tube in a laminar flow cabinet.

3.3. DIGE Experimental Design.

1. Start with a preliminary gel. All experiments should start with a preliminary gel on representative samples to ensure equivocal protein amounts between samples, and that the highest resolution and sensitivity are obtained before embarking on a multigel DIGE experiment (*see* **Notes 13** and **6**). The preliminary gel will also show any problems with the sample preparation that may be corrected by adjusting the procurement methods (*see* **Subheading 3.1**). This step can also be used to optimize the maximal amount of protein can be loaded without adversely affecting resolution.

   The preliminary gel needs only to test one or two of the samples of a much larger experiment. This gel can simply be stained with a total protein stain (*see* **Subheading 2.2.6**) to visually inspect the resolution and sensitivity. Alternatively, the gel can contain two different samples prelabeled with Cy3 and Cy5 and coresolved (*see* **Note 14**).
2. Choose a suitable pH gradient for IEF. Precast isoelectric focusing strips are commercially available from several vendors. The widest length is currently 24 cm, providing the highest resolving power for a given pH range. Medium-range isoelectric focusing gradients (e.g., pH 4–7) offer the best trade-off between overall resolution and sensitivity. Subsequent experiments can then be designed to resolve proteins in the basic range (pH 7–11) and in narrow pI ranges with commensurate increases in protein loading to gain access to the lower abundant proteins in a given sample (*see* **Note 5**). In this way a more comprehensive picture of the proteomes under study can be obtained.
3. Incorporate a pooled-sample mixture internal standard on every DIGE gel in a coordinated experiment. This internal standard, usually labeled with Cy2, is composed of an equal aliquot of every sample in the entire experiment, and therefore represents every protein present across all samples in an experiment. The use of this pooled-sample internal standard on every DIGE gel in a coordinated experiment allows for the facile comparison of independent sample replicates with increased statistical confidence. This experimental design also enables the simultaneous quantitative comparison between multiple variables in a coordinated experiment (**Fig. 1**).
4. Plan out which samples will be labeled with which dyes ahead of time. For minimal dye-labeling chemistry (*see* **Subheading 3.4**), each gel will contain two individual

samples labeled with either Cy3 or Cy5, and an equal amount of the pooled-sample internal standard. The example outlined in **Table 1** is for a two-component comparison repeated in triplicate, with 300 µg total protein loaded onto each of three gels. In this case, 150 µg of each sample should be precipitated (*see* **Subheading 3.2**), resuspended in L-buffer and then split 2:1. Two-thirds of each sample (100 µg) will be individually labeled with either Cy3 or Cy5. The remaining 1/3 of each sample will be pooled together and labeled en masse with Cy2 to serve as an internal standard. By following this, there will be enough of the Cy2-labeled internal standard to have an equal amount as the Cy3 or Cy5 samples loaded onto each gel (*see* **Note 15**).

3.4. CyDye Labeling

All steps are performed on ice. The following protocol is for sample loading via rehydration of IPG strips, and assumes incorporation of a pooled-sample internal standard to coordinate many samples across multiple DIGE gels simultaneously. The steps are summarized in **Table 1** (*see* **Note 16**).

1. Resuspend precipitated sample in 24 µL labeling (L) buffer. Remove 8 µL (1/3 of sample) and place into a new tube that will contain the pooled-sample internal standard (8 µL from all of the other individual samples will be pooled into this tube) (*see* **Note 17**).
2. CyDyes are purchased as dry solids and should be reconstituted to 10x stock solutions (1 nmol/µL) in fresh DMF. Dilute stock solutions of CyDyes 1:10 in fresh DMF to a final working concentration of 100 pmol/µL. (*see* **Note 11**).
3. Label each sample (50–250 µg) with 2–4 µL (200–400 pmol) of either Cy3 or Cy5 working dilution for 30 min on ice in the dark. Label the pooled-sample mixture with 2–4 µL (200–400 pmol) of Cy2 working dilution for every equivalent amount of sample present in the pooled standard as compared with the individually labeled samples. That is, if 100 µg of each sample is labeled with 200 pmol of Cy3 or Cy5, then 50 µg of each of these samples is present in the pooled standard, and 200 pmol of Cy2 is used for every 100 µg of pooled standard (*see* **Table 1** and **Note 18**).
4. Quench reactions with 2 µL of 10 mM lysine for 10 min on ice in the dark.
5. For each gel, combine the quenched Cy3-and Cy5-labeled samples and add 1/3 of the quenched Cy2-labeled pooled mixture.
6. To each tripartite mixture, add an equal volume of 2x R-buffer and incubate on ice 10 min. 2x R-buffer is R-buffer supplemented with an additional 2 mg/mL DTT using the 200 mg/mL DTT stock solution. DTT is omitted from the L-buffer to prevent unfavorable interaction with the CyDyes. Adding an equal volume of 2x R-buffer to the quenched reactions provides the reducing agents to the total reaction volume at 1x final concentration.
7. Add R-buffer (1x DTT concentration) to a final volume suggested by the manufacturer for the given IPG strip length (e.g., 450 µL for 24 cm strips). Add the appropriate volume of IPG buffer ampholines to 0.5% final (v/v) for isoelectric focusing. Proceed with rehydration of dehydrated immobilized pH gradient (IPG)

strips for >16 hr and proceed with isoelectric focusing (*see* **Subheading 3.5.3** and **Note 19**).

3.5. 2-D Gel Electrophoresis and Poststaining

As a result of the minimal labeling, quantification with the CyDyes is carried out on only 2–5% of the proteins that are labeled, and the labeled portion of the protein may migrate at a higher apparent molecular mass than the majority of the unlabeled protein due to the added mass and hydrophobicity of the dyes (exacerbated in lower M_r species). To ensure that the maximum amount of protein is excised for subsequent in-gel digestion and mass spectrometry, minimally labeled 2-D DIGE gels are poststained with a total protein stain (*see* **Subheading 2.2.6**). Accurate excision is also ensured by preferentially affixing the 2nd dimension gel to a presilanized glass plate during gel casting so that the gel dimensions do not change during the analysis. (*see* **Notes 20** and **21**)

These methods assume the use of the Ettan 2-D electrophoresis system (GE Healthcare), but are easily adaptable to other commercially available systems. It also assumes usage of high resolution 24 cm × 20 cm gels.

1. Special gels for 2nd dimension SDS-PAGE. Using low-fluorescence glass plates, pretreat one plate for each gel with 3–5 mL bind silane working solution, carefully wiping the entire surface of the plate with a lint-free wipe. Leave treated plates covered with lint-free wipes for several hours to allow for sufficient out-gassing of fumes (that may contain bind silane) before assembling gel plates and casting of 2nd dimensional SDS-PAGE gels (*see* **Note 22**).
2. Assemble plates and pour 12% homogeneous SDS-PAGE gel(s) using the appropriate amount of 30% stock acrylamide and 4x separating gel buffer for the volumes needed for the number of gels being poured (*see* **Note 23**). Overlay the gels with water-saturated butanol for several hours to provide a straight and level surface to place the focused IPG strip (*see* **Note 10**).
3. Perform isoelectric focusing (for example using an IPGphor III isoelectric focusing unit, GE Healthcare) of the combined tripartite-labeled samples, brought up to final volume with 1x R-buffer and passively rehydrated into immobilized pH gradient (IPG) strips for >16 hr (*see* **Subheading 3.4**, **step 7**) (*see* **Note 24**).
4. Equilibrate the focused IPG strips into the 2nd dimensional equilibration buffer. During this step, the cysteine sulfhydryls in the focused proteins are reduced and carbamidomethylated by supplementing the equilibration buffer with 1% DTT for 20 min at room temperature, followed by 2.5% iodoacetamide in fresh equilibration buffer for an additional 20 min room temperature incubation (*see* **Note 25**).
5. Place equilibrated IPG strip on top of the SDS-PAGE gels that were precast with low-fluorescence glass plates. Use a thin card or ruler to carefully tamp down the IPG strip to the SDS-PAGE gel, removing air bubbles at the interface (*see* **Notes 26** and **27**).

6. Perform 2nd dimensional SDS-PAGE at constant wattage, using << 1 W/gel for at least 1 h prior to ramping up to < 15 W/gel (*see* **Note 28**).
7. CyDye images are acquired using a fluorescence imager, such as the Typhoon 9400 series (GE Healthcare) equipped with lasers and filters that are compatible with the emission/excitation spectra of the dyes. Imaging is performed through the glass plates using the intact gel cassette (*see* **Note 29**).
8. After imaging the gels, carefully remove the plate that was untreated with bind silane. The gel will remain stuck to the treated plate, and can be stained with an appropriate total-protein dye (*see* **Subheading 2.2, item 6**) "open-faced" in the fixation/staining solutions. For SyproRuby, fix gels for at least 2 hr with fixation solution sufficient to completely cover the gel. Longer fixations are possible without adversely affecting subsequent mass spectrometry. After removing the fixation solution, stain gels overnight in SyproRuby and acquire images using a fluorescence imager (*see* **Notes 21** and **30**).

3.6. DIGE Analysis

3.6.1. Software Algorithms

Many bioinformatics tools are commercially available for the comparison of multiple 2-D gel-separated protein spot patterns. Some free internet-based utilities (e.g., www.lecb.ncifcrf.gov/flicker/) provide simple alternation between two spot-patterns, whereas most of the commercial products contain proprietary algorithms for protein-spot detection, inter-gel matching, protein spot quantification and even utilities for building web-based tools for data dissemination. Many include the ability to average replicate patterns into a single virtual pattern to be used in a comparative study. They are all designed to compare multiple spot patterns and quantify abundance changes for individual proteins between experimental conditions.

Several software packages allow for the analysis of DIGE data; the DeCyder suite of software tools were specifically developed to support the DIGE platform when this technology was first marketed by GE Healthcare, and is therefore used as an example here. The DIA (Differential In-gel Analysis) module of DeCyder is used for direct quantification of protein spot volume ratios between the triply codetected signals emanating from each resolved protein, and can be used for the simplest form of a DIGE experiment for pair-wise comparisons with $N = 1$. It is recommended that these DIA-only experiments should be used for test purposes only. The more advanced and statistically significant DIGE experiments that use the internal standard to cross-compare replicate samples from pair-wise and multivariable analyses ($N > 3$) are handled by the BVA (Biological Variation Analysis) module of DeCyder. In a BVA experiment, the signals emanating from the internal standard are used both for direct quantification within each DIGE gel in a coordinated set (using DIA module), as well as for normalization and protein spot-pattern matching between gels (*see* **Note 31**). This allows for the calculation of Student's t-test and ANOVA statistics for individual abundance changes (*see* **Subheading 3.6.2**, and **Table 2**). BVA is also used to match patterns between SyproRuby- and CyDye-stained images to facilitate protein excision for subsequent mass spectrometry (*see* **Notes 20, 21,** and **30).**

3.6.2. Experimental Design and Statistical Confidence.

In the simplest form of a DIGE experiment, two or three samples are separately labeled with one of the three dyes and separated in the same gel for direct pair-wise comparisons. In this case, the software first normalizes the entire signal for each Cy-dye channel and then calculates the protein spot volume ratio for each protein pair. A normal distribution is modeled over the actual distribution of protein pair volume ratios, and two standard deviations of the mean of this normal distribution represent the 95th percent confidence level for significant abundance changes.

This $N = 1$ type of experiment has limited statistical power, since the 95th percentile confidence interval is determined based on the overall distribution of changes within the population (*see* **Note 32**). Many more changes in abundance of much lesser magnitude can be detected with much greater statistical confidence (Student's t-test and ANOVA, **Table 2**) by incorporating independent replicate samples into the experiment (*see* **Note 33**). The number of replicates required in a study depends on the amount of variation in the system being investigated. Increasing the number of replicates will increase confidence in smaller changes in expression. The number of gel replicates that are needed for the experiment to have sufficient sensitivity to detect expression changes can be determined using power calculations ***(41).***

With replicate samples, the Student's t-test and ANOVA statistics are measuring the significance of the variation of a specific protein change, independent of the overall distribution of abundance changes in the population. Incorporating replicate samples into the experimental design also controls for unexpected variation introduced into the samples during sample preparation. This design not only allows for the identification of abundance changes that are consistent across multiple replicates of an experiment, but can also identify significant abundance changes that would not have been identified even if the analyses were performed using Cy3- and Cy5-labeled samples on the same gels, but without the pooled-sample internal standard to coordinate them ***(8).***

3.6.3. Multivariate Statistical Analysis

Univariate analyses such as the Student's t-test and ANOVA (analysis of variants) have traditionally been used in DIGE experiments to provide a list of statistically significant changes in protein abundance. The application of multivariate

Table 2
Statistical Applications of DeCyder Biological Variation Analysis (BVA) and Extended Data Analysis (EDA) modules

Average ratio	Calculated for each protein spot-feature between two groups or experimental conditions. Derived from the log standardized protein abundance changes that were directly quantified within each DIGE gel relative to the internal standard for the protein spot-feature.
Student's T-test	Univariate test of statistical significance for an abundance change between two groups or experimental conditions. P-values reflect the probability that the observed change has occurred due to stochastic chance alone. With DIGE, p-values of <0.01 are often observed. Assumes normal distributions of protein abundance. Can be performed either unpaired or paired.
One-Way ANOVA	Tests for differences in standardized abundance of a given protein across all groups of a multicomponent analysis. Indicates that one group is significantly different from the other groups in the experiment.
Two-Way ANOVA	Tests for differences in standardized abundance of a given protein between multiple groups with the same condition, where multiple conditions are analyzed.
Principal Component Analysis (EDA only)	Reduces the dimension of the variables in a multidimensional space. The first principal component (PC1) divides the dataset along an axis describing the most variance in a system, with the orthogonal second component (PC2) accounting for the second greatest source of variation.
Hierarchical Clustering (EDA only)	Compares groups based on similarity of the collective expression patterns of individual proteins, often displayed in an expression matrix (heatmap). Similarity between groups is proportional to the lateral distance depicted as a branched dendrogram,
K-means (EDA only)	Used to classify proteins into a predefined number of bins based on similarity.
Self Organizing Maps (EDA only)	Similar to K-means, but also clusters nearest neighbors (based on exression patterns) in a 2-D map.
Gene Shaving (EDA only)	Used to identify groups of proteins that have similar expression profiles. Unlike K-means, proteins can belong to more than one group provided there is high coherence within each group.
Discrimnant Analysis (EDA only)	Identifies proteins that can discrimate between groups based on a variety of classifier schemes, including cross validation, feature selection, partial least squares, K-nearest neighbors.

statistical analyses (as outlined in **Subheading 1.2.4**) allow for the assessment of changes on a global scale, and can bring added insight to the usual "list of proteins" generated. Most software packages allow for the export of raw and normalized protein spot-volumes to allow for these additional statistical tests and data manipulations; in addition the DeCyder suite of software tools now provides an Extended Data Analysis (EDA) module that includes many of these tools **(Table 2).** These tools are now becoming more evident in recent DIGE publications ***(5, 18, 22–24, 26, 42).*** Although these multivariate analyses are especially beneficial when analyzing a DIGE experiment that contains three or more conditions, they can also useful in two-condition comparisons to detect sample outliers, fouled samples or even poor experimental design.

Fig. 2 illustrates an example of PCA applied to a DIGE dataset comprised of four experimental conditions each measured in quadruplicate. PCA simplifies multidimensional datasets by reducing the variation down to the two or three most significant sources of variation. In this example, the first principle component (PC1) accounts for 62.3% of the variation amongst 156 proteins of interest, with the second principle component (PC2) accounting for an additional 12.5% of the variation. Each sample datapoint describes the collective expression profile for the subset of 156 proteins, and PC1 and PC2 orthogonally divide the samples into quadrants based on these two largest sources of variation within DIGE dataset. In this case, 75% of the variance between these proteins clusters the samples into the proper categories (adapted from ***(18)***).

Fig. 3 is taken from a 2-D DIGE study which determined the change in protein abundance in mouse liver over a 24-hr period. In this study proteins were harvested from groups of mice on a second cycle after transfer from synchronized light (12 hr light : 12 hr dim red light) to free running conditions (constant dim red light). Proteins were extracted from each liver and pooled from six mice per four-hr time point. Hierarchical clustering (by average distance correlation) was used to investigate the expression of 49 novel circadian proteins. This gave a range of phase groups with 10 proteins peaking during the subjective day and 39 proteins distributed between two clusters which were most abundant during the subjective night (adapted from ***(26)***).

Finally, additional information may be gleaned by mapping proteins found to be changing by DIGE to existing biological pathways and networks. Many software solutions and services are becoming available for this type of extended analysis. Although additional validation is necessary to establish biological significance, the mapping of members of a "list of proteins" to established pathways and networks can provide validating support for the proteins observed by DIGE alone. In some cases, it can also indicate potential proteins associated with the biological question(s) that were not accessible in the DIGE analysis. For example, Friedman et al. ***(5)*** recently reported the use of network/pathway mapping for proteins found by DIGE/MS in MCF10A cells overexpressing the HER2 receptor after treatment with TGF-β. The majority of proteins identified with DIGE/MS mapped to a network of pathways involving TGF-β1 as a major hub, but also included an intercalating pathway involving p53 that effected many proteins that were independently identified in the DIGE/MS experiments. This insight linking new players to those identified with DIGE/MS led to the further investigation of a direct role for p53 in the expression of the tumor suppressor maspin ***(43).***

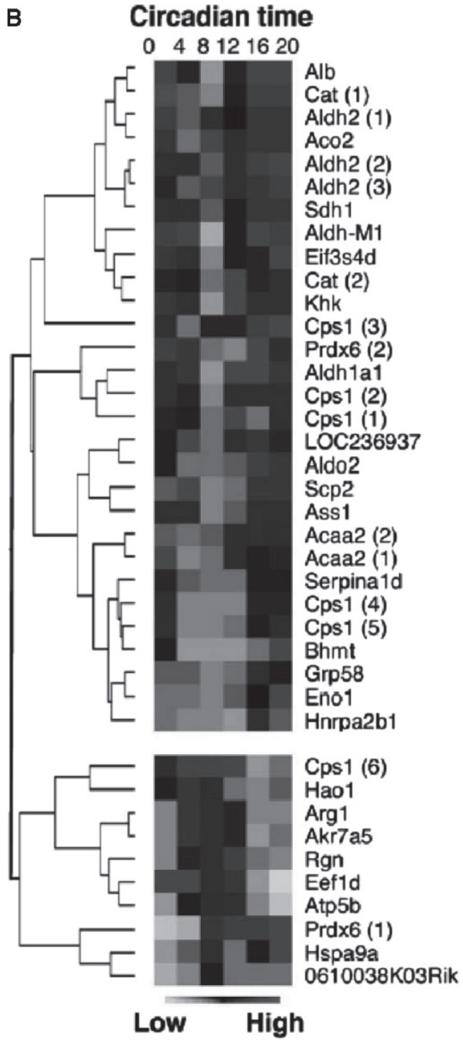

Fig. 3. Hierarchical clustering (by average distance correlation) of representative novel circadian proteins detected by 2-D-DIGE of soluble protein extracts from mouse liver. Pale gray represents low levels of protein expression, black represents intermediate levels, and dark gray represents high levels of expression. (From ***26***).

4. Notes

1. 2-DE has traditionally been a popular method for differential-expression proteomics on a global scale, but until recently, these strategies lacked the ability to directly quantify abundance changes in the same fashion as in stable isotope LC/MS-based strategies ***(44–46)***. This has been mainly due to the inability to directly correlate migration patterns and protein staining between gel separations (gel-to-gel variation). Stable isotopes have been used in gel-based proteomics as well, whereby different proteomes have been separately labeled with different stable isotopes (e.g., growing cells using 14N vs. 15N-labeled medium) prior to mixing and running together through the same 2-DE separation ***(47)***. In this case, abundance changes can be monitored during the mass spectrometry stage on individual proteins, but requires the in-gel digestion and mass spectrometry on every protein present to discover the subset of proteins that is changing.
2. Both hydrophobicity and molecular weight influence how proteins migrate during SDS-PAGE, yielding information on apparent molecular mass.
3. In comparison, commonly used silver or colloidal coomassie blue (c. 5–10 ng sensitivity) stains typically exhibit a dynamic range of less than two orders of magnitude (***4***, ***48***). The CyDye labeling system is compatible with the downstream processing commonly used to identify proteins via mass spectrometry and database interrogation, which involves the generation of tryptic peptides within excised gel plugs. Trypsin cleaves the peptide bonds the C-terminal side of lysine and arginine residues, but peptide generation is mostly unhindered as so few lysine residues are modified by dye labeling.
4. DIGE experiments can still be performed using the internal standard methodology with only two CyDyes, but twice as many gels are required to analyze the same number of samples compared with the three-dye minimal labeling scheme. With saturation labeling, one dye is used to label the internal standard, and the other is used to label individual samples. A dye-swap scheme is not necessary in this case because the individual samples are always labeled with the same CyDye.
5. The use of hydroxyethyl disulfide (commercially available as "DeStreak reagent"), combined with anodic cup-loading, should be used for enhanced resolution for isoelectric focusing above pH 8 (***49***).
6. Running every DIGE gel with the maximal amount of protein (without adversely effecting first-dimension resolution) not only enables detection of lower abundance proteins, but also provides more material for subsequent protein identification using mass spectrometry. This makes every gel in a coordinated DIGE experiment a "pick-able" gel, without the need to run subsequent preparative gels with increased protein load that then have to be carefully matched to a lower-abundant, analytical gel. When combined

with narrow range isoelectric focusing, maximizing the protein amount also allows interrogation of the lower abundant proteins in a sample.

7. If one sample within a study has very skewed protein distributions compared with others, then many of the "novel proteins" within this sample will effectively be diluted out in the pool. Such a sample outlier can be easily identified using the multivariate statistical analyses described.
8. Repetition not only enables the identification of subtle differences with statistical confidence, it is also vital to control for non-biological variation. In most cases biological variation will outweigh technical variation, therefore only biological replicates are necessary. Thus it is important that each replicate sample is derived from an independent experiment, ideally performed on different occasions as perhaps using different batches of medium. The independent samples can then be analyzed coordinately using the pooled-sample internal standard methodology. See Table 1 for an example of this design.
9. All solutions should be prepared using water that has a resistivity of 18.2 MΩ-cm; this is referred to as "water" throughout the text.
10. Mix equal parts butanol and water and shake vigorously. Let the two phases separate overnight, and use the butanol phase for overlay. Butanol that is not completely water-saturated can extract water from the top of the gel. A more recent improvement is to use a 0.1% SDS solution in a conventional spray bottle, used to carefully spray a fine mist over the top of the gels to thoroughly cover the top of the gel (the gel/overlay interface will not be as obvious).
11. DMF can degrade, producing amines which can react with the NHS-ester CyDyes. DMF stocks should be kept fresh (< 3 months) and anhydrous to ensure optimal labeling conditions.
12. For buffers that contain urea, care should be taken to ensure the urea is fresh and free of the natural breakdown product isocyanate, which will carbamylate free amines and thereby neutralize the protonatable epsilon-amine groups of lysines residues. This is problematic for several reasons, the foremost being the fact that this gives rise to artificial charged-train isoforms in the first dimension isoelectric focusing. Heating samples above 37°C should also be avoided, as this facilitates the conversion to isocyanate. Any buffer component which contains a primary amine, such as pharmalytes, ampholytes, HEPES buffers etc. should be avoided as these components may react with the CyDyes thus reducing their affective concentration.
13. For example, 500 μg of material may be loaded onto a pH 4–7 24 cm IPG strip), but due to the overall distribution of proteins in the sample, as well as a sometimes unusually high abundance of a subset of proteins, may result in much less material actually resolving between the electrodes. A good rule to follow is to load the desired amount based on the protein concentrations, and then adjust the load by eye as necessary.

14. This is DIGE in its most simplistic form, and can show differences between the samples without interference from gel-to-gel variation, but provides limited statistical power to help distinguish true biological variation from background such as artificial noise introduced during sample preparation.
15. Employing a dye-swapping approach will control for any dye-specific effects that may result from preferential labeling or different fluorescence characteristics of acrylamide at the different wavelengths of excitation for Cy2, Cy3 and Cy5, especially at low protein spot volumes. This is easily incorporated into any DIGE analysis where repetitive samples are used (along with the internal standard to compare across multiple DIGE gels).
16. For saturation chemistry, general methods and considerations are the same as for the minimal chemistry, but there are several unique features to also consider for the saturation chemistry. First, careful optimization of the labeling conditions must be carried out for each new sample set to ensure complete reduction of cysteine residues. Insufficient labelling will lead to multiple protein features in the second dimension due to MW and hydrophobicity shifts. Overlabeling results in side reactions with the epsilon-amine groups of lysine side chains, but since the maleimide dyes do not carry compensatory charge, this results in the overall loss of a charge which creates a series of isoelectric forms in the first dimension ("charge trains"). Labeling buffer should not contain any components with free thiols, as these will react with the satCyDyes.
17. L-buffer volume can be increased if necessary for complete resolubilization, although 100–250 μg or more should resolubilize readily in this volume. The volume of labeling buffer used for resolubilization should not exceed 40 μL per sample when using cup-loading for sample entry to ensure that the final volumes will not exceed the capacity of the cup-loading (c. 100–150 μL).
18. These methods are provided assuming that all gels to be run will be used both for analytical (quantification) as well as preparative (providing material for subsequent mass spectrometry) purposes. Current recommendations from the manufacturer are to label 50 μg of sample with 400 pmol CyDye. Sufficient amount of unlabelled sample can be added to the quenched reactions to achieve final protein amounts to facilitate subsequent mass spectrometry. Alternatively, many have found that the ratios can be adjusted to label increasing amounts of sample (up to 200 μg with 200 pmol dye) without adversely affecting the overall labeling reaction (presented here).
19. If samples are to be introduced using anodic cup-loading, simply bring this mixture up to 100 μL in R-buffer and proceed with cup-loading. R-buffer can always be supplemented with additional DTT using the 200 mg/mL DTT stock solution. In the presence of Destreak reagent for focusing in pH ranges above pH 8, the addition of equal volume 1x R-buffer should provide sufficient amount of DTT without interfering with the Destreak reagent.

20. Comparison of minimally labeled protein 2-D maps with unlabeled protein maps is generally not a problem, as the addition of only one dye molecule does not generally prevent the facile matching of small alterations in protein mobility between the 2–5% labeled protein and the remaining unlabeled protein that will provide enough material for mass spectrometry.
21. Poststaining is not necessary with Saturation DIGE, since an unlabeled population with potentially different migration characteristics will not exist.
22. This treatment binds the gel to one of the glass plates and therefore prevents shrinking/swelling during the poststaining and protein excision processes, thereby facilitating accurate robotic protein excision. Nothing should be placed on top of wipes that are covering bind silane-treated plates, as this may leave impressions that are detected during the scanning phase. Assembly and casting too soon may create a binding surface on the opposite glass plate, preventing the gel to be subsequently poststained and picked. Automated protein excision can be facilitated for certain systems by placing fluorescent alignment reference targets on the plate, which can be performed at this stage.
23. A stacking gel is not required for 2-D gel electrophoresis, as the proteins are effectively "stacked" to the height of the IPG strips. SDS is also not essential in the separating gel, as the SDS associated with the proteins during the equilibration step, and present in the running buffer, is sufficient (although many traditionally use it in the separating gel). Using 2x concentration running buffer in the upper buffer chamber can produce higher quality separations in some circumstances.
24. Samples of similar nature should always be focused simultaneously for optimal reproducibility. Focusing programs vary for some pH gradients. A typical program for many ranges is 500 V for 500 V-hr, stepping to 1000 V for 1000 V-hr, followed by a final step to 8000 V until > 50 V-hr have been reached. Check recommendations from specific vendors. Using the ceramic manifold tray adaptor is recommended.
25. Volume of equilibration buffer should be large to ensure sufficient removal of ampholines and other components of the 1st dimensional run.
26. Carefully wash out any remaining liquid on top of the SDS-PAGE gel. Prewet the IPG strip with1x running buffer and place the strip between the gel plates with the plastic-backing adhering to the inside surface of one of the glass plates. The prewetted running buffer will facilitate the manipulation of the IPG strip down the inside surface of the plate and on top of the SDS-PAGE gel.
27. An agarose overlay, used by many protocols, is not absolutely necessary to ensure proper contact between the IPG strip and the 2nd dimensional SDS-PAGE gel. Using a thin card or ruler to carefully tamp down the IPG strip to the gel is usually sufficient and removes the added problems associated with the overlay, such as trapped air bubbles in the solidified agarose.

28. Running gels at less than 1 W/gel can improve resolution in the high molecular weight regions of the 2nd dimension gel. Use wattage appropriate for the 2nd dimensional unit being used. Many different gel units can accommodate increased power by compensating for the increased heat.
29. Absorption/emission maxima in DMF are 491/506 for Cy2, 553/572 for Cy3, and 648/669 for Cy5; although care must be taken to scan in regions of each spectrum that do not contain absorbance or emission in the other spectra, which may mean using a non-maximal region of a given spectrum.
30. Comparison of the 2-D spot maps between saturation-labeled samples and minimal labeled or unlabeled samples is impossible, as proteins containing multiple cysteine residues may appear as significantly larger M_r species when labeled with the saturation dyes, which of course cannot be predicted without first knowing the protein identity.
31. Almost all software packages for 2-D electrophoresis involve matching of protein spot-patterns between gels. For DeCyder, it is used in the BVA module to match the quantitative data obtained from the triply coresolved protein signals from each gel in the DIA module (where gel-to-gel variation does not come into play). Manual verification of the matching is almost always required with any software package.
32. There are many "all-or-none" type of experiments where the single gel comparison may be valid, and subtle changes are not expected. Nevertheless, using independent replicates and the pooled-sample internal standard methodology is still needed to control for non-biological sample preparation error.
33. The multigel approach allows many data points to be collected for each group to be compared. Protein features of interest can be selected by looking for significant change across the groups. Student's t-test and Analysis Of Variance (ANOVA) probability scores (p) indicate the probability that the observed change occurred due to stochastic, random events (null hypothesis). Probability values < 0.05 are traditionally used to determine a statistically significant difference from the null hypothesis. As this represents 50 potential false-positives for 1,000 resolved proteins, confidence intervals within the 99th percentile ($p < 0.01$) are arguably more valid, and can be attained using DIGE (5, 8, 18, 50–54).

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Comparing 2D Electrophoretic Gels Across Internet Databases: An Open Source Application

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1. Introduction

In ***(1–4)*** and in the second edition of this book ***(5),*** we described a Web-based computer-assisted visual method called Flicker for comparing two two-dimensional (2D) protein gel images across the Internet using a Java applet. In the second edition of this book ***(6),*** Flicker was described as a stand-alone application. The flicker method was originally developed in the GELLAB 2D-gel analysis system ***(7–11).*** The applet version was used for Web-based analysis using a Web-browser.

The Java stand-alone application can run on a user's computer, where it can access images from the Web but can easily access the user's images data on their local computer. Flicker is available as open source on http://open2Dprot.sourceforge.net/Flicker where the executable program and the source code is also available. Some of the code was derived from the old Flicker applet program and some from the MicroArray Explorer ***(12)*** –an open-source data-mining tool for microarray analysis http://maexplorer.sourceforge.net/. The Open2Dprot protein expression analysis system http://open2Dprot.sourceforge.net/ was partly derived from Flicker, MicroArray Explorer, and GELLAB code and concepts.

Because Flicker can analyze a user's data on his or her computer independent of the Internet, this gives the user the ability to perform real-time comparisons of 2D-gel image data with gel images residing on the user's local file system, on remote Internet databases on the Internet, or a combination of both sources.

This approach may be useful for comparing similar protein samples created in different laboratories to help putatively identify or suggest possible protein spot identification. The gels should be run under similar pH and molecular weight ranges if possible. Although available for over three decades, 2D polyacrylamide gel electrophoresis (2D-PAGE) is still used ***(13–14)*** even considering

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the now common use of mass spectrometry ***(13,25–26)*** and protein microarrays ***(13,21–22)*** for protein identification and biomarker discovery as part of the researcher's toolkit ***(13).***

Advances, such as IEF "zoom" fractionation gels ***(23),*** are commonly used ***(24)*** to divide the protein sample by pH range or immunoaffinity subtraction with LC ***(18),*** greatly increase the resolution and numbers of spots able to be discriminated by subsequent 2D-gel electrophoresis. Another increasingly common image comparison technique uses 2 to 6 cyanine dyes using dye multiplexing to label multiple control and experimental samples run in the same gel such as GE Healthcare's (formerly Amersham's) DIGE ***(13,25–26)*** and scanned with system's like Perkin Elmer's ProExpress ***(27).*** Multiple scans of the same gel using different color filters can then be color mapped to see the contributions of the different samples. This is useful if one has control over the experimental design when determining the reference gel, set of control gels, and experimental gels. Advances in DIGE (DeCyder™) EDA software now allow comparison of multiple images run on the same apparatus ***(26).*** However, these advances do not completely solve the problem of trying to putatively compare one's own sample against an Internet reference gel where they have identified protein spots.

A number of 2D-gel databases that contain gel images are available on the Web for various types of tissues. Proteins are identified for some of the spots in a subset of these databases. Both WORLD-2DPAGE and 2D Hunt on the http://www.expasy.org/ server can be used to find Web URL addresses for a number of 2D protein gel databases. Google searches are also used and we link to these sites in the Help menu. Many of these databases contain 2D-gel images with identified proteins. Some of these databases let you identify spots in their gels by clicking on a spot in their gel image shown in your Web browser. It then queries their Web server database to determine if the spot you pointed to is in that database and report its identity if found. These "clickable" 2D-gel map images are often published using a common federated database (DB) schema ***(28–29).*** One of the more interesting databases is SWISS-2DPAGE ***(28–31),*** accessible from the Expasy site. It has a large number of tissues with over 30 gel databases including a wide range of human tissues, mouse, *E. coli*, *aribidopsis*, *dictyostelium*, and yeast. Their site also has a series of IEF zoom fractionated gels for *E. coli*.

We have incorporated links to these SWISS-2DPAGE database gel map images so they may be loaded and accessed directly from Flicker after having putatively matched a spot in your gel with one in the SWISS-2DPAGE gels. If you have loaded one of these active map gel images in Flicker and enabled the database access, then clicking on a spot in that image will pop up a Web page as it tries to look it up the spot in the SWISS-2DPAGE database. If a SWISS-2DPAGE data entry exists for the spot coordinates you have selected, then it will report the corresponding protein or tell you it cannot be found.

By comparing one's own experimental 2D-gel image data with gel images of similar biological material from these from Internet reference databases, it opens up the possibility of using the spots in these reference gels to suggest the putative identification of apparently corresponding spots in your gels. The image analysis method described here allows scientists to more easily collaborate and compare their gel image data over the Web.

How Can We Compare Two Gels?

When two 2D gels from different laboratories are to be compared, simple techniques may not suffice. There are several methods for comparing two gel images: (1) put the images side by side and visually compare them; or (2) slide one gel (autoradiograph or stained gel) over the other while backlighted; or (3) build a 2D-gel quantitative computer database from both gels after scanning and quantitatively analyzing these gels using a 2D-gel database system; and (4) run DIGE dye multiplexing ***(25)*** to label different samples in the same gel or across gels ***(26)***. A variant of this last method is to spatially warp two gels to the same geometry and then pseudocolor them differentially. These methods may be impractical for many investigators since in the first case the physical gel or autoradiograph from another laboratory may not be locally available. The first method may work for very similar gels with only a few differences. The second method will work better for gels that are not so similar but that have local regions that are similar. The third method may be excessive if only a single visual comparison is needed because of the costs (labor and equipment) of building a multigel database solely to answer the question of whether one spot is probably the same spot in the two gels. The fourth method may also not be practical if you want to compare your sample against an existing reference gel.

The Flicker Program

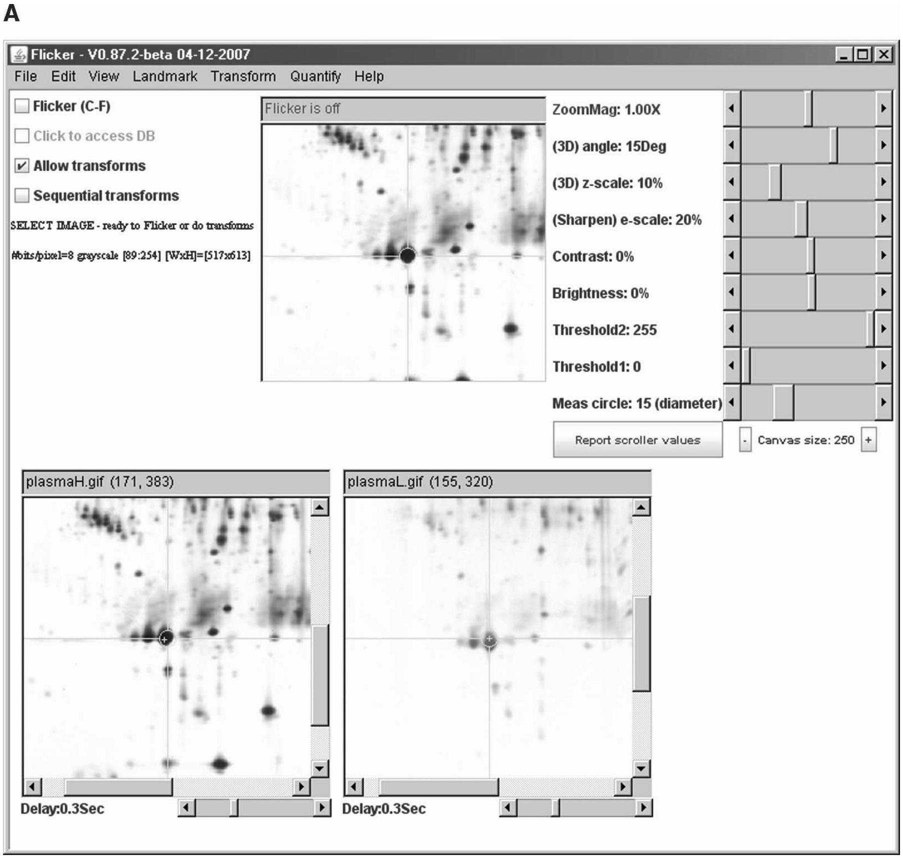
We describe a computer-based image comparison technique called flicker that has been used for years in finding differences in star maps in astronomy.

The Flicker program runs on most computers. It is started as one would any program after it is downloaded from the Flicker server and installed (*see* **Method 1**). One gel image may be read from any Internet 2D-gel database (e.g., SWISS-2DPAGE, etc.), the other may reside on the investigator's Web server where they were scanned or copied, or the two gel images may be from either Web server source.

Figure 1 shows the Flicker application after it has been started with some demo gels. You interact with the program by clicking or dragging the mouse in the left or right images, adjust parameter scrollers (upper right), set interaction modes (checkboxes upper left), keyboard short-cut commands, and primarily pull-down menu commands.

Notation in This Paper

We use the notation (<*menu name*> | <*command*>) throughout this paper to indicate the these menu commands. The <m*enu name*> indicates one of the



☐ Flicker (C-F)
☐ Click to access DB
☑ Allow transforms
☐ Sequential transforms

SELECT IMAGE - ready to Flicker or do transforms

#bits/pixel=8 grayscale [89:254] [WxH]=[517x613]

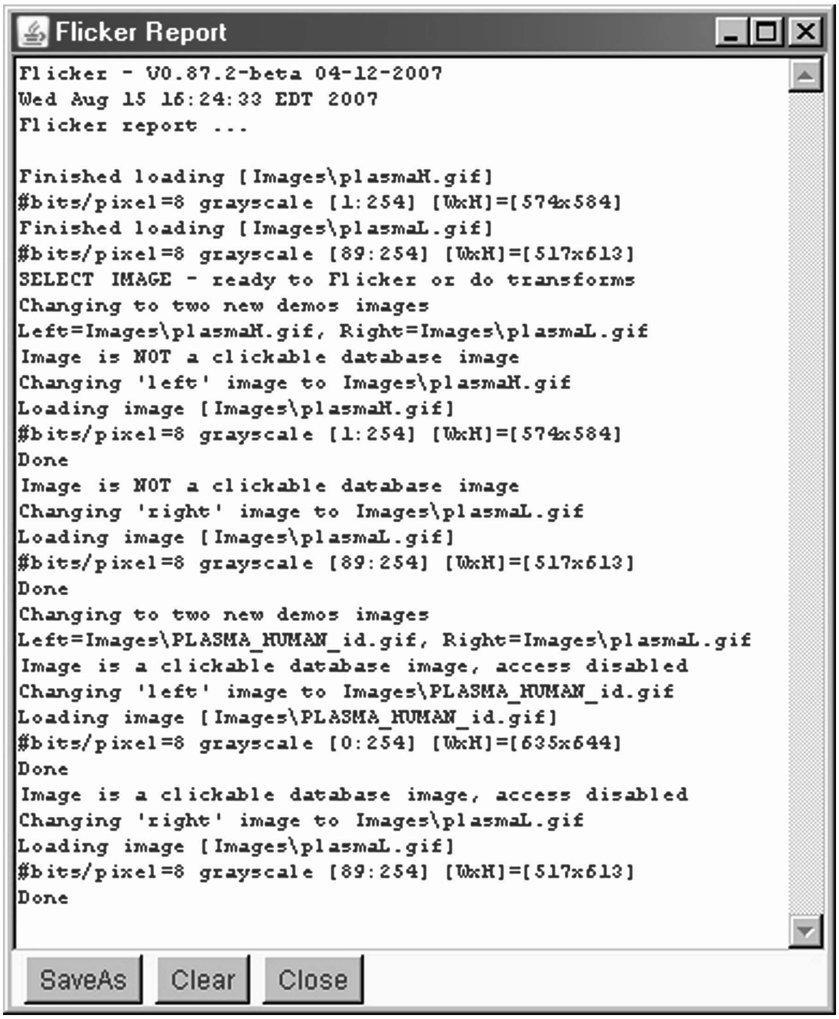
ZoomMag: 1.00X
(3D) angle: 15Deg
(3D) z-scale: 10%
(Sharpen) e-scale: 20%
Contrast: 0%
Brightness: 0%
Threshold2: 255
Threshold1: 0
Meas circle: 15 (diameter)
Canvas size: 250

plasmaH.gif (171, 383)
Delay:0.3Sec

plasmaL.gif (155, 320)
Delay:0.3Sec

Fig. 1. Screen view of initial Flicker program. **(A)** shows the pull-down menus at the top used to invoke file operations, editing, view selection, landmarking, image transforms, spot quantification, and help. A set of scroll bars on the right determines various parameters used in some of the transforms. The File menu options include opening a new gel image. Check-boxes on the left activate flickering and active gel map access if the data supports it. A set of status lines appear below the checkboxes and indicate the state of operation and various other messages. The flicker image is in the upper middle of the frame when it is enabled. The two labeled human blood plasma gel images are shown in the bottom scrollable windows that may be positioned to the region of interest. These windows also have associated flicker time-delays used when flickering. Image *plasmaH* is an IPG non-linear gradient gel from SWISS-2DPAGE in Geneva and *plasmaL* is a carrier-ampholyte linear gradient gel from the Merril Lab at NIMH. Transformed image results are shown in the same scrollable windows. The four checkboxes are: **Flicker** (C-F) to enable/disable flickering; **Click to access DB** checkbox enables/disables access to a Web server that is associated with a clickable image DB if it exists for the selected image; **Allow transforms** checkbox enables/disables image transforms; **Sequential transforms** checkbox enables/disables using the last image transform output as input for the next image transform. The parameters used in various transforms

B



```
Flicker - V0.87.2-beta 04-12-2007
Wed Aug 15 16:24:33 EDT 2007
Flicker report ...

Finished loading [Images\plasmaH.gif]
#bits/pixel=8 grayscale [1:254] [WxH]=[574x584]
Finished loading [Images\plasmaL.gif]
#bits/pixel=8 grayscale [89:254] [WxH]=[517x613]
SELECT IMAGE - ready to Flicker or do transforms
Changing to two new demos images
Left=Images\plasmaH.gif, Right=Images\plasmaL.gif
Image is NOT a clickable database image
Changing 'left' image to Images\plasmaH.gif
Loading image [Images\plasmaH.gif]
#bits/pixel=8 grayscale [1:254] [WxH]=[574x584]
Done
Image is NOT a clickable database image
Changing 'right' image to Images\plasmaL.gif
Loading image [Images\plasmaL.gif]
#bits/pixel=8 grayscale [89:254] [WxH]=[517x613]
Done
Changing to two new demos images
Left=Images\PLASMA_HUMAN_id.gif, Right=Images\plasmaL.gif
Image is a clickable database image, access disabled
Changing 'left' image to Images\PLASMA_HUMAN_id.gif
Loading image [Images\PLASMA_HUMAN_id.gif]
#bits/pixel=8 grayscale [0:254] [WxH]=[635x644]
Done
Image is a clickable database image, access disabled
Changing 'right' image to Images\plasmaL.gif
Loading image [Images\plasmaL.gif]
#bits/pixel=8 grayscale [89:254] [WxH]=[517x613]
Done
```

Fig. 1. (continued) are adjusted directly by first selecting an image and then adjusting its values. You can popup the scrollable report window using the **Report scroller values** button. These parameters are saved when you save the state. You can change the size of the three image windows by using the **"+"** and **"–"** buttons**.** **(B)** The popup scrollable report window shows a log of all text output that appears in the status lines. It may be saved to a text file on the local disk.

pull-down menus: File, Edit, View, Landmark, Transform, Quantify, Help. The <*command*> indicates one of the commands in that menu. **Table 1** summarizes the menu commands. Some of the commands have alternative keyboard shortcuts

Table 1
Summary of Commands Available in Flicker Nenu

Submenus are indicated by underlining the name of the menu and adding the '->' symbol. Checkboxes are indicated by the ☐ prefix to the command. Shortcuts are indicated by a (C-<key>) at the end of the command.

File menu – *to load images, load demo images, active map urls, to load/save the Flicker .flk state, update (from the server): program, DB/Flk*DB.txt database files*

- Open image File – pop up gel image file browser
- Open image URL – pop up gel image URL dialog
- Open demo images -> – load pairs of demonstration gel images from 3 data sets
- Open user images -> – load pairs of demonstration gel images
  - Pairs of images -> – load pairs of user gel images
  - Single images -> – load single user gel image to selected image
  - List user's images by directory
- Open active map image -> – load active gel image from Internet(Swiss-2DPAGE)
- Open recent images -> – load an image you have used recently
- Assign active image URL – to one of the open images to make it active

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- Open state file – restore the Flicker state of previously saved session
- Save state file – save the Flicker state in current .flk state file
- SaveAs state file – save the Flicker state in new .flk state file

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- Update -> – download and update your program and data from server
  - Flicker program – to get the most recent release
  - Active Map Image database – get latest active maps database
  - Demo Images database – get latest demo images database
  - Add user's active images DB by URL

——

- Save Transformed image – of selected image as .gif file if transformed
- SaveAs Overlay image – the current overlay image
- Reset images – to the initial state when they were loaded
- Abort transform – abort any active image transforms

——

- Quit – exit the program, saving the .flk state of Flicker in the process

Edit menu - *to change various defaults*

- Canvas size -> – change the size of 3 image canvases and overall Flicker window
  - Increase size (C-Numpad '+') - increase the canvas size
  - Decrease size (C-Numpad '–') - decrease the canvas size
- Set colors -> – set default colors for the overlays
  - Target colors – to change the target color
  - Trial object colors – to change the trial object color
  - Landmarks colors – to change the color of landmarks
  - Measurement color – to change the color of measurements
  - Guard Region color – to change the color of the guard region

(continued)

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☐ Use linear else log of TIFF files > 8-bits – take log of tiff data if > 8-bits
☐ Enable saving transformed images when do a 'Save(As) state'
☐ Use protein DB browser, else lookup ID and name on active images

——

Auto measure, protein lookup in active server and Web page popup – set access
Select access to active DB – select protein report for active protein queries
    Use SWISS-2DPAGE DB access
    Use PIR UniProt DB access
    Use PIR iProClass DB access
    Use PIR iProLink DB access

——

Reset default view – sets all view options to the defaults
Clear all 'Recent' images entries – clears the list of recently accessed images
View menu - *to change the display overlay and popup report window options*
m Flicker images (C-F) – toggle flickering on and off
Set overlay options – select one or more image overlay options
    ☐ View landmarks – add landmarks to the overlay display in images
    ☐ View target – add target to the overlay display images
    ☐ View trial object – add trial object to the overlay display images
    ☐ View Region Of Interest (ROI) - add ROI to the overlay display images
Set measurement options – select one or more image measurement options
    ☐ View measurement circle – add measurement circles to overlay display images
    ☐ Use 'Circle' for measurement spot locations
    ☐ Use '+' for measurement spot locations
    ☐ Use 'spot number'for spot annotations – e.g., *#1, #2*, …, etc.
    ☐ Use 'spot ID'for spot annotations – e.g., *actin, APO-A1*, etc.
Set gang options – select one or more ganged images (left and right) options
    ☐ Multiple popups – make multiple popup windows instead of reusing one
    ☐ Gang scroll images – move left and right images scrolling together
    ☐ Gang zoom images – zoom left and right images scrolling together
☐ Add guard region to edges of images – this allows comparing edges of images
☐ Display gray values (C-G) – show gray values of cursor trial object
☐ Show report popup – display the report popup window again if needed
Landmark menu - *to define landmarks for spatial image warping*
    Add landmark (C-A) – add trial objects (in images) as landmark
    Delete landmark (C-D) – delete the last landmark defined
    Show landmarks similarity – compute a LSQ error similarity measure of the two sets of landmarks
        Set 3 predefined landmarks for demo images (C-Y)
        Set 6 predefined landmarks for demo images (C-Z)
Transform menu - *contains various image processing transforms for selected image(s)*
    Affine Warp – warp selected image using 3 pairs of landmarks

(continued)

- Pseudo 3D transform – do pseudo 3D scaling based on image intensity
  - ——
  - Sharpen Gradient – gradient + gray scale sharpen selected image
  - Sharpen Laplacian – Laplacian + gray scale sharpen selected image
  - Gradient – gradient of the selected image
  - Laplacian – Laplacian of the selected image
  - Average – average selected image
  - Median – median of selected image
  - Max 3×3 - max of 3×3 neighborhood of selected image
  - Min 3×3 - min of 3×3 neighborhood of selected image
  - ——
  - Complement – complement selected image
  - Threshold – threshold the selected image by gray values in (T1:T2)
  - Contrast Enhance – Contrast enhance selected image
  - Histogram equalize – histogram equalize selected image
  - ——
  - Original color - Restore original data for selected image
  - Pseudo color - compute pseudo color scaling for selected image
  - Color to grayscale - compute NTSC RGB to grayscale transform for selected image (gray = red*0.33 + green*0.50 + blue*0.17)
  - ——
  - Flip Image Horizontally – flip image horizontally selected image
  - Flip Image Vertically – flip image vertically selected image
  - ——
  - Repeat last transform (C-T) - repeat last transform, if any
- ☐ Use threshold inside (T1:T2) filter - filter by pixels inside the range (T1:T2), otherwise pixels outside of (T1:T2))

Quantify menu - *contains OD calibration, background and foreground measurements*

- Measure circle -> – measure intensity/density within circle
  - Capture background (C-B) – background measurement at current position
  - Capture measurement (C-M) – report object measurement at current position
  - Circle size -> – define the circlular mask size by radius in m 1×1 to m 11×11 pixels subregions
  - ——
  - Clear measurement – clear measurement data
  - Edit selected spot(s) ‘id’ fields from spot list(s) (C-I)
  - Edit selected spot(s) from spot list(s) (C-E)
  - Delete selected spot from spot list (C-K)
  - ——
  - List spots in the spot list for selected image – list measured spots
  - List spots in the spot list (tab-delimited) – same but can cut& paste data
  - List ‘id’-paired annotated mean norm. spots in both spot lists (tab-delim.)
  - List ‘id’-paired annotated spots in both spot lists (tab-delimited)

(continued)

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Lookup Protein Ids & Names in spot list from active map server (sel'td image)

——

Clear spot list (ask first) for selected image

Print data-window -> - print numeric pixel data around image pixel
  Show data window of selected pixel (C-V)
  Set print window size – either 5×5, 10×10, … or 40×40
  Set print data radix – either decimal, octal, hex, or optical density

Calibrate -> - Calibrate grayscale as optical density or other units
  Optical density by step wedge – set calibration by ND step wedge in gel image
  Use demo Leukemia ND step wedge preloaded data – for use in demo

  ——

  Optical density by spot list – set OD values by mean spot list measurements

Region of Interest (ROI) -> - region of interest operations
  Set ROI ULHC (C-U) - define upper left hand corner of ROI
  Set ROI LRHC (C-L) - define lower right hand corner of ROI
  Clear (ROI) (C-W) - delete ROI
  Show ROI grayscale histogram (C-H) – show grayscale histogram of ROI pixels
  Capture measurement by ROI (C-R) - measure integrated density inside the computation region ROI. Use the circular mask mean background for background correction

☐ Use sum density else mean density - use sum of the density else mean density within the region

☐ List-of-spots else trial-spot measurement mode (C-J)

Help menu - *popup Web browser documentation on Flicker from the server*
  Flicker Home – pop up Flicker home page open2Dprot.sourceforge.net/Flicker
  Reference Manual – pop up the reference manual for Flicker application
  How-to use controls -> – pop up the reference at particular sections
  Vignettes -> – pop up short vignettes showing how-to-do tutorials
  Version on the web site – show current version available on the Web site
  About – show details on Flicker application

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Book chapter on Flicker (2005) – popup 2005 PDF of book chapter

Old flicker applet documentation ->
  Flicker applet home page – popup the home page for old Flicker applet
  EP97 Paper – popup the Electrophoresis '97 paper Flicker applet
  Poster 1 – pop up poster describing Flicker applet
  Poster 2 – pop up poster describing Flicker applet
  Poster 3 – pop up poster describing Flicker applet
  Poster 4 – pop up poster describing Flicker applet

2D gel web resources ->
  SWISS-2DPAGE – pop up SWISS-2DPAGE home page
  WORLD-2DPAGE – pop up Expasy World-2DPAGE of federated gel databases
  2D-HUNT – pop up Expasy's 2D-Hunt for finding gel Web sites
  Google 2D search – pop up Google search for finding gel Web sites

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activated by using the *Control key* with another key and are indicated as Control-<*key*> or C-<*key*> (e.g., C-A). The checkbox menu commands are indicated with a □ prefix. Checkbox commands may be toggled on and off.

The gels in the two lower left and right images are specified by the user with the FlickerFiles menu. Gel images may be loaded from: the local computer (File | Open image file), or any Internet site with GIF, JPEG, TIFF or PPX images (with .gif, .jpg, .tiff or .tif, or .ppx GELLAB-II format ***(9)*** file extensions) using the (File | Open image URL) command. In addition, the installation provides a few demonstration images (File | Open demo images) that loads pairs of comparable images. You may also specify active gel images from Web servers as described below.

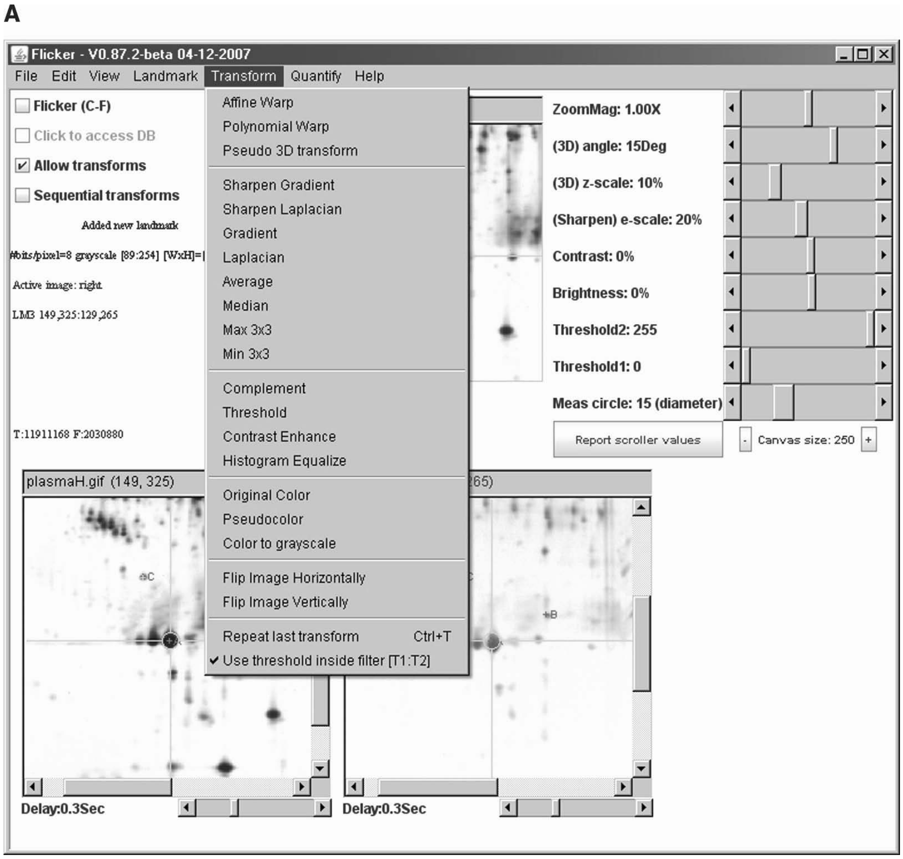
When Flicker starts, it creates additional submenu entries in the (File | Open user images | Pairs of images | …) and (File | Open user images | Single images | …) submenus that are the names of the user's images they copied to the Flicker Images/ subdirectory. The user can load images using those commands.

Flicker is also capable of interacting with federated 2D-gel databases to retrieve data on individual protein spots if one of the gels is a federated gel having an associated clickable gel map database. After aligning gels in Flicker, you enable federated database access in Flicker and then click on a spot in the gel belonging to the federated database (*see* **Fig. 2**). This causes a Web page to pop up with information from the federated server describing that protein. We provide menu entries (File | Open active map image | …) to let you load one of the SWISS-2DPAGE gel images.

You may load a gel image in the lower left or right image windows. First click on the image you want to load the new image. Then select the active gel image to you want using the entry (File | Open active map image | …) pull-down menu (e.g., select (… | SWISS-2DPAGE Human | Plasma)). Next, click on the other image and then use the other gel image you want to compare it with using either (File | Open image file) or (File | Open image URL) commands.

The Flicker program is written in Java, a general purpose, object-oriented programming language developed by Sun Microsystems ***(32)*** http://java.sun.com/. Java has become a standard for portable Internet Web applications.

Most often, the original images may be compared directly. However, occasionally, the comparison may be made visually easier by first applying enhancement transforms such as spatial warping, brightness, contrast or other image transforms. Adjusting image brightness and contrast so the two gels have similar ranges will make the image fusion easier for the user when flickering. For gels with a lot of geometric distortion, it is useful to adjust the geometry of one gel so that the geometry of the local region being compared approximates that of the other gel. By local geometry, we mean the relative positions, distances, and angles of a set of spots in corresponding regions.


A
Flicker - V0.87.2-beta 04-12-2007
File Edit View Landmark Transform Quantify Help
Affine Warp
Polynomial Warp
Pseudo 3D transform
Sharpen Gradient
Sharpen Laplacian
Gradient
Laplacian
Average
Median
Max 3x3
Min 3x3
Complement
Threshold
Contrast Enhance
Histogram Equalize
Original Color
Pseudocolor
Color to grayscale
Flip Image Horizontally
Flip Image Vertically
Repeat last transform Ctrl+T
✓ Use threshold inside filter [T1:T2]
Report scroller values
- Canvas size: 250 +
plasmaH.gif (149, 325)
265)
C
B

☐ Flicker (C-F)

☐ Click to access DB

☑ Allow transforms

☐ Sequential transforms

Added new landmark

#bits/pixel=8 grayscale [89:254] [WxH]=

Active image: right

LM3 149,325:129,265

T:11911168 F:2030880

ZoomMag: 1.00X

(3D) angle: 15Deg

(3D) z-scale: 10%

(Sharpen) e-scale: 20%

Contrast: 0%

Brightness: 0%

Threshold2: 255

Threshold1: 0

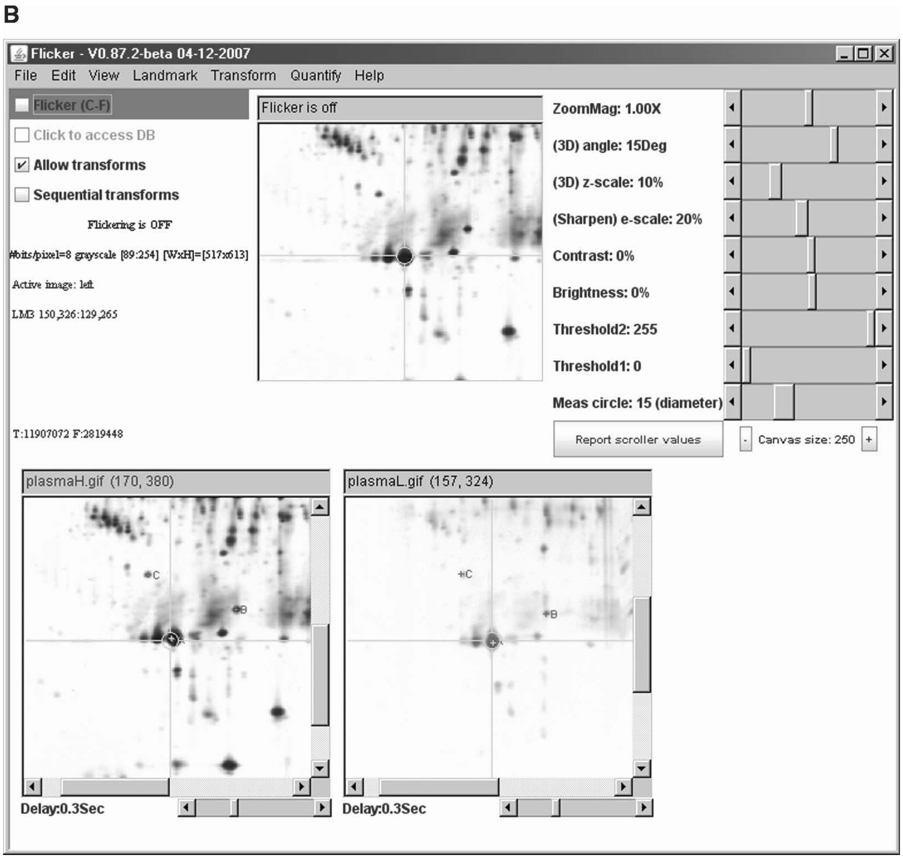
Meas circle: 15 (diameter)

Delay:0.3Sec

Delay:0.3Sec

Fig. 2. Screen view of the landmarks used for the affine transform of the human plasma gel images. **(A)** The transform warps the geometry of a local region defined by the three landmarks so it more closely resembles the geometry of the corresponding local region in the other gel. Scrollable image windows with 3 "active" landmarks defined in both gel images that were selected interactively in preparation for doing the affine image transform. Corresponding landmark spots are selected so they are defined unambiguously in both gel images. For demonstration purposes, the command (Landmarks | Set 3 predefined landmarks for demo gels) will set up the 3 landmarks shown in this figure. **(B)** After defining the 3 landmarks, use the (Transform | Affine warp) command.

One technique to correct geometry differences is called spatial warping. When performing spatial warping, corresponding regions of interest are **(1)** first marked by the user (we call this "landmarking") with several corresponding points in each gel image (3 for affine warping and 6 for poly warping), and **(2)** then one of the two gel images is warped to the geometry of the other gel



☐ Flicker (C-F)
☐ Click to access DB
☑ Allow transforms
☐ Sequential transforms

Flickering is OFF

#bits/pixel=8 grayscale [89:254] [WxH]=[517x613]

Active image: left

LM3 150,326:129,265

T:11907072 F:2819448

Flicker is off

ZoomMag: 1.00X
(3D) angle: 15Deg
(3D) z-scale: 10%
(Sharpen) e-scale: 20%
Contrast: 0%
Brightness: 0%
Threshold2: 255
Threshold1: 0
Meas circle: 15 (diameter)

Canvas size: 250

plasmaH.gif (170, 380)

plasmaL.gif (157, 324)

Delay:0.3Sec

Delay:0.3Sec

Fig. 2. (continued)

(see equations 1 and 2). A landmark is a corresponding spot that is present in both gels. Landmarks are defined by clicking on the spot to mark and selecting the (Landmark | Add Landmark (C-A)) command. The warping is performed by first selecting the image to warp by clicking on it, and then and selecting the (Transform | Affine Warp) command. Landmarking and warping are described in more detail below.

Spatial warping doesn't change the underlying grayscale values of the synthesized warped image to the extent that would cause local structural objects to appear and disappear and thus spot artifacts might be created. Instead, it samples pixels from the original image to be transformed and places them in the output image according to the geometry of the other input image. After warping is finished, gels may then be compared visually by flickering.

1.1. Image Flickering

The basic concept of using flickering as a dynamic visualization technique is simple. If two images may be perfectly aligned then one could simply align them by overlaying one over the other and shifting one image until they line up. However, many images such as 2D PAGE gels have rubber-sheet distortion (i.e., local translation, rotation, and magnification). This means there is more distortion in some parts of the image than in others. Although it is often impossible to align the two whole images at one time, they may be locally aligned piece-by-piece by matching the morphology of local regions.

If it appears that a spot and the surrounding region do match, then one has more confidence that the objects are the same. This putative visual identification is our definition of matching when doing a comparison. Full identification of protein spots requires further work such as cutting spots out of the gels and subjecting them to sequence analysis, amino-acid composition analysis, mass spectrometry, testing them with monoclonal antibodies, or other methods.

2.2. Image Enhancement

It is well-known that 2D gels often suffer from local geometric distortions making perfect overlay impossible. Therefore, making the images locally morphologically similar while preserving their grayscale data may make them easier to compare. Even when the image subregions are well aligned, it is still sometimes difficult to compare images that are quite different. Enhancing the images using various image transforms before flickering may also help. Some of these transforms involve spatial warping, which maps a local region of one image onto the geometry of the local region of another image while preserving its grayscale values. Another useful operation is contrast enhancement that helps when comparing light or dark regions by adjusting the dynamic range of image data to the dynamic range of the computer display. Other transforms include image sharpening and contrast enhancement. Image sharpening is performed using edge enhancement techniques such as adding a percentage of the gradient or Laplacian edge detection functions to the original grayscale image. The gradient and Laplacian have higher values at the edges of objects. In all cases, the transformed image replaces the image previously displayed. Other functionality is available in Flicker and is described in the **Methods** and **Notes** subheadings of this chapter, **Table 1,** and on the Web server.

1.3. Image Processing Transforms

As mentioned, there are a number of different image transforms that may be invoked from the Transform menu. You may display the transformed image, use it as input to another transform, or save it as a .gif file on your local computer. When you save the state, you may also save the transformed images.

1.3.1. Affine Spatial Warping Transform

The spatial warping transforms require defining several corresponding landmarks in both gels. As we mentioned, one gel image can be morphologically transformed to the geometry of the other using the affine or other spatial warping transformations. These transforms map the selected image to the geometry of the other image. It does not interpolate the gray scale values of pixels – just their position in the transformed image. As described in **(1–2, 4–6),** this might be useful for comparing gels that have some minor distortion, comparing local regions, gels of different sizes or gels run under slightly different conditions. Flicker uses the affine transform as an inverse mapping as described in ***(33).*** Let $(u_{xy}, v_{xy}) = f(x,y)$, where (x,y) are in the output image, and corresponding (u,v) are in the input image. Then, in a raster sweep through the output image, pixels are copied from the input image to the output image. The affine transformation is given in **Eqs. 1–2:**

$$u_{xy} = ax + by + c \tag{1}$$

$$\nu_{xy} = dx + ey + f \tag{2}$$

When the affine transform is invoked, Flicker solves the system of 6 linear equations for coefficients (a,b,c,d,e,f) using three corresponding landmarks in each gel.

1.3.2. Pseudo-3-D Transform

As described in **(1–2, 4–6),** the Pseudo-3-D transform is a forward mapping that generates a pseudo 3-D relief image to enhance overlapping spots with smaller spots seen as side peaks. The gel size is width by height pixels. The gray value determines the amount of y shift scaled by a percentage z_{scale} (in the range of 0 to 50%). Pseudo perspective is created by rotating the image to the right (left) by angle theta (in the range of –45 to +45 degrees). The transform is given in **Eqs. 3–5** for image of size width X height, shift in the horizontal dimension computed as d_x.

$$\mathrm{d}_x = \mathrm{width}\ \sin(\theta) \tag{3}$$

$$x' = (d_x\ (\mathrm{hight} - \mathrm{y})/\mathrm{hight}) + x \tag{4}$$

$$y' = y - \mathrm{z}_{scale} * g(x,y) \tag{5}$$

where $g(x,y)$ is in the original input image and (x',y') is the corresponding position in the output mapped image. Pixels outside of the image are clipped to white.

The Pseudo-3-D transform is applied to both images so that one can flicker the transformed image.

1.3.3. Edge Sharpening

Edge sharpening may be useful for sharpening the edges of fuzzy spots. The sharpened image function $g'(x,y)$ is computed by adding a percentage of a 2-dimensional edge function of the image to original image data $g(x,y)$ as shown in equation **(6).** The edge function increases at edges of objects in the original image and is computed on a pixel by pixel basis. Typical "edge" functions include the 8-neighbor gradient and Laplacian functions that are described in **(1–2, 4–6)** in more detail. The e_{scale} value (in the range of 0 to 50%) is used to scale the amount of edge detection value added.

$$g'(x,y) = (e_{\mathrm{scale}} * \mathrm{edge}(x,y) + (100 - e_{scale}) * g(x,y))/100. \quad (6)$$

2. Materials

The following lists all items necessary for carrying out the technique. Since it is a computer technique, the materials consist of computer hardware, software and an Internet connection. We assume the user has some familiarity with computers and the World Wide Web.

1. A Windows PC, MacIntosh with MacOS-X, a Linux computer or a Sun Solaris computer having a display of at least 1024 × 768 resolution. At least 30 Mb of memory is required and more is desirable for comparing large images or performing many transforms. If there is not enough memory, it will be unable to load the images, the transforms may crash the program or other problems may occur. An Internet connection is required to download the program from the http://open2Dprot.sourceforge.net/Flicker Web site (*see* **Note 1**). New versions of the program will become available on this Web site and can be uploaded to your computer using the various Update commands described in the Notes section or from the Downloads Web page. If you will be using the active gel image maps associated with federated 2D-gel databases, then you *will* need the Internet connection for accessing those (e.g., SWISS-2DPAGE) databases. You do *not* need the Internet for local image comparisons.
2. You should have Java (JDK or JRE version 1.5.0 or later) installed already on your computer. If you do not, you can download it from java.sun.com for free and install it yourself.
3. When you install Flicker, it creates several subdirectories: *Images/* (containing the demonstration images), *DB/* (containing startup database files), and *FlkStartups/* (containing any startup files you create when you do a (File | SaveAs state file). The DB/ files are: *FlkDemoDB.txt* which describes the demo images, *FlkMapDB.txt* which describes the gel images and their corresponding active image map URLs, and

FlkRecentDB.txt which lists recently accessed images. An empty database file *FlkRecentDB.txt* contains the file names and active gel map URLs, if any, of the recently accessed images. The program is in *Flicker.jar* is the Java Archive File for Flicker that is executed when you run Flicker. It also uses *jai_core.jar*, the core Java runtime from SUN's Java Advanced Imaging (JAI at java.sun.com) and *jai_codec.jar* that is the JAI tiff file reader from SUN's Java Advanced Imaging JAI at reader. For Windows users, there is a Flicker.exe file that you can click on to run the program. Otherwise, there are two script files *Flicker-startup.bat* (for Windows) and *Flicker-startup.sh* for Unix/Linux or Mac OS-X that run it through the Java interpreter.

4. The Internet is a good source of 2D-gel images. You can find them by searching WORLD-2DPAGE and 2D Hunt on the http://www.expasy.org/ server or a Google search to find other Web 2D protein gel image databases. Links to these databases are available in the (Help | 2D gel Web resources) submenu.
5. We currently distribute Flicker so that it uses up to 96 megabytes of memory. If you want to run it with more or less memory, you will need to edit the startup files *Flicker-startup.bat* or *Flicker-startup.sh* you use to set it to a value in the range of 30 Mb to 1784 Mb. Both of these startup files contain the command

```
java -Xmx96M -jar Flicker.jar
```

So to increase the startup memory, change the 96 M to some larger value (e.g., to increase it to 500 megabytes, change -Xmx96M to –Xmx500M).

3. Method

We now describe the operation of the Flicker from the user's point of view. You first install Flicker. Then run it with either the demonstration images, your own images or images from the Internet. Then you simply flicker the gel images. If necessary, to improve the image comparability, use image enhancement transforms first before then flickering the two images.

3.1. Installing Flicker from the Web Server

Click on the *Download* link on the http://open2Dprot.sourceforge.net/Flicker Web site. The following method can be used to download the Zipped Flicker-dist installation package and install it as described below.

Go to the Web site's *Files mirror* under the Flicker releases. Look for the most recent release named "*Flicker-V.XX.XX-dist.zip*" such as *Flicker-V0.87.2-dist.zip*. These zipped files include the program, required jar libraries, demo data, and Windows batch and Unix shell scripts. Download the zip file and put the contents where you want to install the program. Note that there is a *Flicker.exe* for Windows (created with launch4j.sourceforge.net). You might make a short-cut to this file to make it easier to find when starting the program. Alternatively, you can use the sample *Flicker-startup.bat* or *Flicker-startup.sh* scripts to run the program explicitly via the java interpreter. Note that this method assumes that you have Java installed on your computer and that it is at *least* JDK (Java Development

Kit) or JRE (Java Runtime Environment) version 1.5.0. If you don't have this, you can download the latest version free from the java.sun.com Website.

The following is a short procedure that summarizes the procedure for downloading and installing Flicker.

1. In the Table of Contents on the left on the home page, click on "Files mirror" under "Source Code".
2. Under latest file releases, where it has the header "Package", click on the "open2Dprot" below that.
3. This will refresh the page and if you scroll down, it will show "Flicker files".
4. Select the "+" on this to list the files. Pick the one with the highest version number called something like *Flicker-V0.87.2-dist.zip* file.
5. Click on that to download it.
6. Put it where you want to install it and unpack it. There is a *Flicker.exe* file (for Windows) as well as .bat and .sh file scripts.
7. If you have images to compare, you can copy them or subfolders of images into the Image folder in the distribution directory.

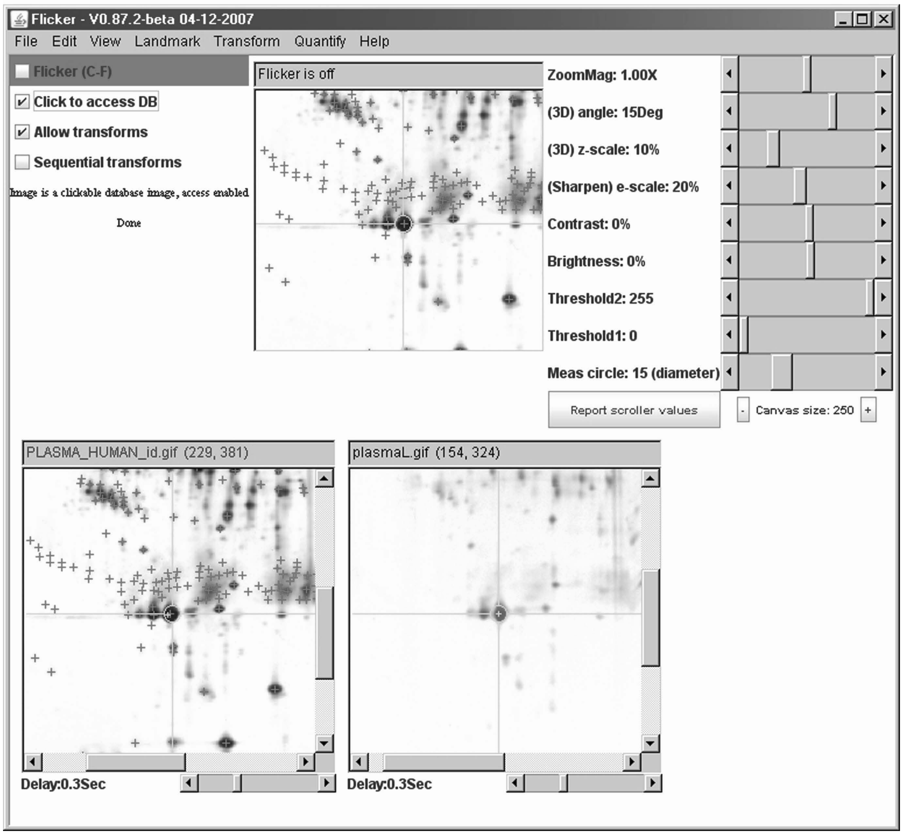
To start Flicker in Windows, click on the startup icon for *Flicker.exe*. You can also start it in Windows by clicking on the *Flicker-startup.bat* file. For Unix systems including MacOS-X, you can start Flicker from a command line file by specifying the path to *Flicker-startup.sh*. Normally it comes up with the two demonstration human plasma 2D-gel images (*plasmaH.gif* - an IPG gel from SWISS-2DPAGE on the left) and (*plasmaL.gif* – a carrier ampholyte gel from Dr. Carl Merril/NIMH on the right).

If you have your own gels (JPEG, GIF or TIFF formats), you can try loading them. But first you must copy the files or folders of files to the *Images/* folder where you installed Flicker. Then use the (File | Open user images | …) commands to load the images. There is currently no way to load images stored in a relational database. You may want to limit resolution by first decreasing their size using an image editing program like Adobe PhotoShop, shareware program ThumbsPlus (www.cerious.org), or open source programs like Gimp (www.gimp.org). Large very high-resolution images that are 20 Mb to 40 Mb will not work well. We suggest reducing the size to about 1 K×1 K for good interactivity if you have any problems with running out of memory or very sluggish response. These image-editing programs can also be used for converting other formats to JPEG, GIF or TIFF formats that Flicker can read.

3.2. Graphical User Interface for Flickering

Fig. 1 shows the initial screen of the Flicker program. Pull-down menus at the top invoke file operations, edit preferences, view overlay options, landmarking, image transforms, and help commands. Scroll bars on the side determine various parameters used in the transforms. The two images to be compared are

A


Flicker - V0.87.2-beta 04-12-2007
File
Edit
View
Landmark
Transform
Quantify
Help
Flicker is off
Report scroller values
Canvas size: 250
PLASMA_HUMAN_id.gif (229, 381)
plasmaL.gif (154, 324)

☐ Flicker (C-F)
☑ Click to access DB
☑ Allow transforms
☐ Sequential transforms

Image is a clickable database image, access enabled

Done

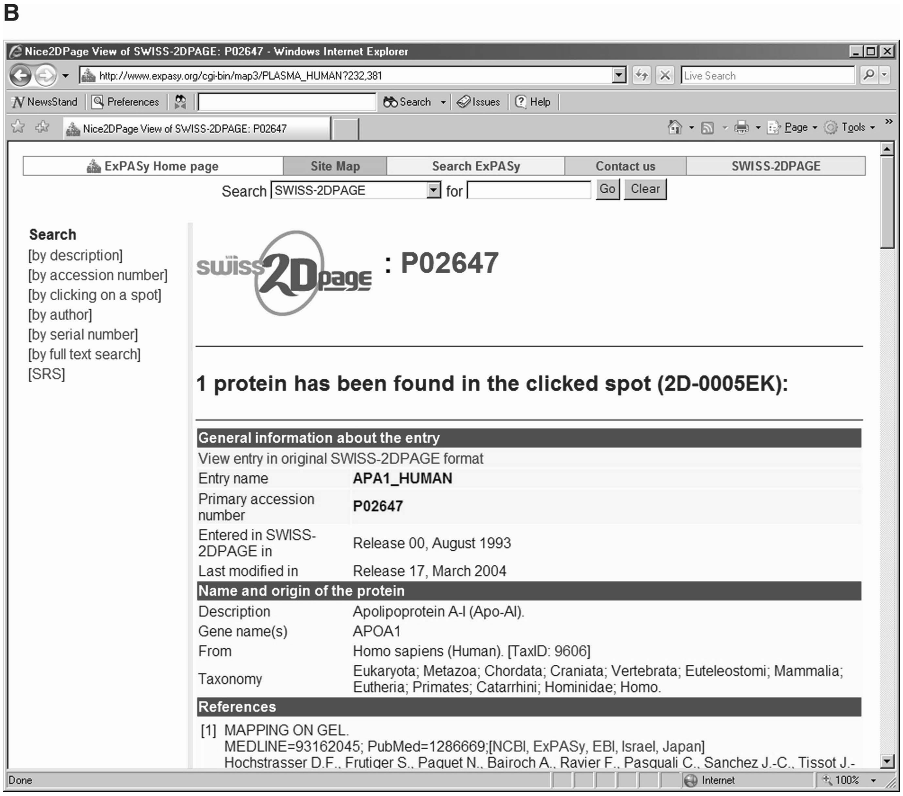
ZoomMag: 1.00X
(3D) angle: 15Deg
(3D) z-scale: 10%
(Sharpen) e-scale: 20%
Contrast: 0%
Brightness: 0%
Threshold2: 255
Threshold1: 0
Meas circle: 15 (diameter)

Delay:0.3Sec

Delay:0.3Sec

Fig. 3. Screen views of clickable active gel compared with another gel. **(A)** We have loaded the active SWISS-2DPAGE human plasma gel (*PLASMA_HUMAN_id*) in the left image and the *plasmaL* gel in the right image. Spots that appear in SWISS-2DPAGE are indicated with red "+" symbols. We then aligned the images using flickering. We then selected the **Click to access DB** checkbox. Finally, we clicked on the indicated spot in the left gel to determine the putative identification of the corresponding spot in the right gel. **(B)** The SWISS-2DPAGE window then popped up as a result of clicking on that spot in the left image and indicates the putative protein identification of the visually corresponding spot in the right gel. The *plasmaH* image is the same gel as *PLASMA_HUMAN_id* but without the graphic overlays. You can load these same gels using the (File | Open demo images | Human Plasma gels | (SWISS-2DPAGE vs Merril) - clickable) command, which should be used when you are connected to the Internet.

loaded into the lower scrollable windows. A flicker window appears in the upper middle of the screen. Checkboxes on the left activate flickering and control display options. A group of status lines below the checkboxes indicate the state


B
Nice2DPage View of SWISS-2DPAGE: P02647 - Windows Internet Explorer
http://www.expasy.org/cgi-bin/map3/PLASMA_HUMAN?232,381
Live Search
NewsStand
Preferences
Search
Issues
Help
Nice2DPage View of SWISS-2DPAGE: P02647
Page
Tools
ExPASy Home page
Site Map
Search ExPASy
Contact us
SWISS-2DPAGE
Search SWISS-2DPAGE for
Go
Clear
Search
[by description]
[by accession number]
[by clicking on a spot]
[by author]
[by serial number]
[by full text search]
[SRS]
swiss 2Dpage
Done
Internet
100%

: P02647

1 protein has been found in the clicked spot (2D-0005EK):

General information about the entry	
View entry in original SWISS-2DPAGE format	
Entry name	**APA1_HUMAN**
Primary accession number	**P02647**
Entered in SWISS-2DPAGE in	Release 00, August 1993
Last modified in	Release 17, March 2004
Name and origin of the protein	
Description	Apolipoprotein A-I (Apo-AI).
Gene name(s)	APOA1
From	Homo sapiens (Human). [TaxID: 9606]
Taxonomy	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
References	

[1] MAPPING ON GEL.
MEDLINE=93162045; PubMed=1286669;[NCBI, ExPASy, EBI, Israel, Japan]
Hochstrasser D.F., Frutiger S., Paquet N., Bairoch A., Ravier F., Pasquali C., Sanchez J.-C., Tissot J.-

Fig. 3. (continued)

of operations. **Table 1** shows the summary of the commands in the pull-down menus.

Only part of an image is visible in the scrollable windows. These subregions are determined by setting horizontal and vertical scroll bars. Another, preferred, method of navigating the scrollable images is to click on the point of interest while the CONTROL key is pressed. This will re-center the scrollable image around that point. This lets the user view any subregion of the image at high resolution. These images may be navigated using either the scroll bars or by moving the mouse with the button pressed in the scrollable image window. Then, each image in the flicker window is centered at the point last indicated in the corresponding scrollable image window.

Note that if you are near the edge of the image when you do this, it will may not scroll the image properly. To fix this problem you can add a “guard region” around the edge of the image using the (View | ☐ Add guard region to edges of images). This is useful for aligning spots that are along the edge of the images while flickering.

The guard region's color can be changed via the Edit menu (Edit | Set colors | Guard region color). The guard region requires more memory that is why the default is to have the guard region default to off.

A flicker window is activated in the upper-middle of the screen when the "Flicker" checkbox is selected. Images from the left and right scrollable images are alternatively displayed in the flicker window. The flicker delay for each image is determined by the adjusting the scroll bar below the corresponding scrollable image window. Various graphic overlays may be turned on and off using the various view "overlays" selected in the (View | <sub-menus> …) checkbox menu commands.

Clicking on either the left or right image selects it as the image to use in the next transform. However, clicking on the flicker image window indicates the next transform you might use should be applied to *both* left and right images. You can change the selected image by just clicking on any of the images.

You can increase or decrease the size of the three image windows by using the (Edit | Canvas size | Increase size (C-keypad '+')) and (Edit | Canvas size | decrease size (C-keypad '–')) commands. This will resize the main window accordingly. Alternatively, and easier to do, is to click on either the left-arrow (shrink) or right-arrow (enlarge) buttons on the **Canvas size** scroller on the middle right edge of the Flicker application window.

3.3. Loading Images

As mentioned in the introduction, gel images may be loaded into the left or right selected image from: (a) the local computer using the (File | Open image file) command or (b) any Internet site using the (File | Open image URL) command. You may load pairs of demonstration images that come with Flicker, and are installed in the *Images/* directory. Use the (File | Open demo images | …) command to load them into the left and right images. The demos include a few samples that may be useful for initially learning the system. They include: two human plasma gels - an IPG SWISS-2DPAGE gel vs a carrier ampholyte gel (Merril/NIMH); some human leukemias (AML, ALL, CLL, HCL) from Lester et.al. ***(10–11).***

You may specify active gel images from the Web using the (File | Open active map image **| …**) to let you load one of the Swiss-2DPAGE gel images into the left or right selected image. This list of active images is defined by the tab-delimited *FlkMapDB.txt* file read by Flicker when it is started. "Power users" could edit this file (use Excel and save as tab-delimited) to add active map entries pointing to other federated 2D-gel Web databases. The *FlkMapDB.txt* file is provided with your download installation in the *DB/* directory.

Gel images are loaded into the lower left or right images. First click on the left or right image you want to replace. Then, select the active gel image to you want using the entry (File | Open active map image | …) pull-down menu (e.g., select (… | SWISS-2DPAGE Human | Human Plasma)). Next, click on the other image and then open the other gel image you want to compare it with using either (File | Open image file) or either (File | Open image URL) commands.

3.4. Adding Your Own Image Data to the User Images/Database

There is another way for users to add many of their gel images without editing the *DB/FlkDemoDB.txt* file. When you place your image data directories in the *Images/* directory, Flicker will discover them when it starts and add them to the demo menu. It works as follows:

1. You copy or move one or more of your directories of with the images you want to use with Flicker in the *Images/* folder.
2. When Flicker starts, it creates additional submenu entries in the (File | Open user images | Pairs of images | …) and (File | Open user images | Single images | …) submenus that are the names of the user's directories.
3. The first submenu contains unique combinations of pairs of all images within each of the user's directories. Selecting one of these entries will load the pair of images into the left and right Flicker image windows.
4. The second menu command lets you select the right or left Flicker image, and then load a single image from any of the user image directories into that Flicker image window. This would be useful if you wanted to compare one of your images with one of the Internet reference gels.

3.4.1. Flickering

When flickering two images with the computer, one aligns putative corresponding subregions of the two rapidly alternating images. The flicker window overlays the same space on the screen with the two images and is aligned by interactively moving one image relative to the other using the cursor in either or both of the lower images. Using the mouse, the user initially selects what they suspect is the same prominent spot or object in similar morphologic regions in the two gel images. The images are then centered in the flicker window at these spots. When these two local regions come into alignment, they appear to pulse and the images fuse together. At this point, differences are more apparent and it is fairly easy to see which spots or objects correspond, which are different, and how they differ. We have found that the user should be positioned fairly close to the flicker window on the screen to optimize this image-fusion effect (i.e., it does not work as well standing back more than a few feet from the screen).

3.4.2. Selecting the Proper Time Delays When Flickering

The proper flicker delays, or time each image is displayed on the screen, is critical for the optimal visual integration of image differences. We have also found that optimal flicker rates are dependent on a wide variety of factors including: amount of distortion, similarity of corresponding subregions, complexity and contrast of each image, individual viewer differences, phosphor decay-time of the display, ambient light, distance from the display, and so on. We have found the process of flickering images is easier for some people than for others.

When comparing a light spot in one gel with the putative paired darker spot in the other gel one may want to linger longer on the lighter spot to make a more positive identification. Because of this, we give the user the ability to set the display times independently for the two images (typically in the range of 0.01 second to 1.0 second with a default of 0.30 second) using separate Delay scroll bars located under each image. If the regions are complex and have a lot of variation, longer display times may be useful for both images. Differential flicker delays with a longer delay for the light gel are useful for comparing light and dark sample gels. This lets you stare at the lighter spots to have more verification that they are actually there.

3.5. Image Processing Methods

As mentioned, there are a number of different image transforms that may be invoked from the menus. These are useful for changing the geometry, sharpness, or contrast making it easier to compare potentially corresponding regions. As we go through the transforms we will indicate how they may be used. Some affect one image while some affect both. Flickering is deactivated during image transforms to use most computational power for doing the transforms.

The TRANSFORM menu has a number of commands that include warping, grayscale transforms and contrast functions. The two warp method selections: “Affine Warp and “Poly Warp” are performed on only one image (the last one selected by clicking on an image). The “Pseudo-3-D” transform makes a 3-D image with the “peaks” created proportional to gray level. Unlike the warp transforms, the grayscale transforms are performed on both images. These include: “SharpenGradient”, “SharpenLaplacian”, “Gradient”, “Laplacian”, “Average”, “Median”, “Max3×3” and “Min3×3”. The contrast functions are “Complement” and “ContrastEnhance”. You can transform image color images to grayscale using the “Color to grayscale” command, and generate a false color image from a grayscale using the “Pseudo color” command. You can flip the image using “Flip image horizontally” or “Flip image vertically” commands.

3.5.1. Landmarks: Trial and Active

The affine transform requires that three active landmarks be defined before it can be invoked. A trial landmark is defined by clicking on an object’s center anywhere in a scrollable image window. This landmark would generally be placed on a spot. Clicking on a spot with or without the CONTROL key pressed still defines it as a trial landmark. After defining the trial landmark in both the left and right windows, selecting the (Landmark | Add Landmark (C-A)) command to define them as the next active landmark pair and identifies them with a red letter label (+A, +B, +C, …) in the two scrollable image windows. The (Landmark | Delete Landmark (C-D)) command is used for deleting the last landmark you defined.

3.5.2. The Affine Transform for Spatial Warping

The two warping transforms, affine (see **Eqs. 1** and **2**) and polynomial, require 3 and 6 landmarks respectively. Attempting to run the transform with insufficient landmarks will cause Flicker to notify you that additional landmarks are required. The image to be transformed is the one last selected. You must select either the left or right image. **Fig. 2a** shows the landmarks the user defined in the two gels before the affine transform. **Fig. 2b** shows the transformed image. Then, recenter the transformed image before you flicker. After the transform, the landmarks can be lined up perfectly, and adjacent spots will line up better.

3.5.3. Pseudo-3-D transform

As described in **(1–2, 4–6)** and as shown in **Eqs. 3–5,** the Pseudo-3-D transform generates a pseudo 3D relief image to enhance overlapping spots with smaller spots seen as side peaks. The gray value determines the amount of "*y*" shift scaled by a percentage (set by scroll bar z_{scale} (in the range of 0 to 50%). Pseudo perspective is created by shifting the image to the right or left by setting by scroll bar "angle" degrees (in the range of –45 to +45 degrees). Negative angles shift it to the right and positive angles to the left. The image to be transformed is the one last selected. If neither was selected (i.e., you clicked on the flicker window), then both images are transformed.

3.5.4. Edge Sharpening

Edge sharpening may be useful for improving the visibility of the edges of fuzzy spots. You can select either a Gradient or Laplacian edge sharpening function using the "SharpenGradient" or "SharpenLaplacian" operation in the TRANSFORM menu where the image to be transformed is the one last selected. The Laplacian filter generates a "softer" edge than the Gradient. You can set the scroll bar $\mathrm{e}_{\mathrm{scale}}$ value (in the range of 0 to 50%) to scale the amount of edge detection value added. The image to be transformed is the one last selected. If neither was selected (i.e., you clicked on the flicker window), then both images are transformed.

3.5.5. Putative Identification of a Spot in One Gel by Comparison with Federated Database Gel Map

Open an active gel image in the lower left or right window. First click on the window you want to load the new image. Then, select the active gel image to you want using the entry (File | Open active map image | …) pull-down menu (e.g., select (… | SWISS-2DPAGE Human | Plasma)). Next, click on the other image and then use the other gel image you want to compare it with using one of the other (File | Open …) commands

At this point flicker the two images so that you can make a putative guess on which spot you are interested in your gel corresponds to which spot in the active map gel. Then shut off flickering by turning off the “Flicker” checkbox. Then turn on the “Click to access DB” checkbox. Then click on the spot in the active map image which will pop up a Web browser window indicating the SWISS-2DPAGE web page for that spot if it is in their database.

4. NOTES

1. Status of the Flicker application.

As of the time of the submission of this chapter, most of the functionality available in the former Java applet (1–2, 4–6) is fully functional in the stand-alone application. The current state of Flicker is documented on the Web server. A future release of Flicker will contain a spot quantification capability with the ability to calibrate the image (either from the calibration information in the image itself if available, or from a scanned neutral density step wedge scanned with the image), estimate background density, and estimate spot intensity with a background subtraction. Documentation is available on the Web site. This documentation may also be invoked from the “Help” submenus.

The original Flicker program was converted from a Java applet to a Java application by Peter Lemkin and Greg Thornwall with help from Jai Evans. Code was added from the open source MicroArray Explorer (http://maexplorer.sourceforge.net/) program. The new Flicker program uses the Mozilla 1.1 open source license and is available on the open source Web server http://open2Dprot.sourceforge.net/Flicker/.

You can update your program and data files using the various Update options in the Files menu. The (File | Update | Update Flicker Program) command downloads and installs the latest *Flicker.jar* file. The (File | Update | Update active Web maps DB) command downloads and installs the latest active Web maps database *DB/FlkMapDB.txt* file. The (File | Update | Update Demo images DB) command downloads the latest demo images into the *Images/* directory.

2. Hints on measuring spots.

There are some disadvantages in comparing gels visually. It is useful for doing a rough comparison and there is currently no simple way available to do adequate quantitative comparison (as can be done with existing 2D-gel computer database systems) that use automatic spot segmentation and global normalization methods. However, you can look at the gray value of the cursor in the left or right image if you enable the (View | μ Display gray values in image title (C-G)) menu option. The program also allows single-spot quantification with optical density calibration. These limitations should be kept in mind when using the technique.

In the mean time, if your gels and scanner are reasonably linear so that grayscale approximates protein concentration, you can use the simple grayscale method that can be used for the ballpark estimates of density. You can do this either of two ways: by measuring the area under a circular mask (you can set the radius), or the area inside a rectangular Region of Interest (ROI). Note that unless the spot fits well inside of the mask or ROI, you will not get a very accurate measurement. Both methods can subtract an optional background value you can capture and so can give intensity corrected for background if defined.

To set the measurement circle region size (1×1, 3×3, 5×5 …, or 11×11), select the image you will work on and the adjust the Meas Circle slider on the right.. Click on a background region near where you want to measure a spot's density within a circular mask. Then select the (Quantify | Measure by circle | Capture background (C-B)) command. Then click on the center of the spot you want to estimate and select the (Quantify | Measure by circle | Capture measurement (C-M)) command. This will compute and display background corrected data that appears in the report window as:

```
plasmaH.gif (201,392) totBkgrd: 1557gray, meanBkgrd: 21 gray
CircleMask: 5X5 area: 73 pixels
(1) plasmaH.gif (214,376)
        Tot(Meas-Bkgrd): 9418.000 gray
        TotMeas: 10975.000 (39.000:227.000) gray
        TotBkgrd: 1557.000 (201,392) (16.000:28.000) gray
        mnDens: 154.577, mn(Dens-Bkgrd): 132.648, mnBkgrd: 21.930 gray
        CircleMask: 5X5 area: 73 pixels
```

The (View | Set measurement view options | ☐ View measurement circle) displays the background and foreground measurement circles if enabled with "**B**" and "**M**" labels. Set the (View | ☐ Use sum density else mean density) menu option to specify that it report either total region density or mean density.

You also can measure intensity inside a rectangular ROI regions you set by using both the (Quantify | Region of Interest (ROI) | Set ROI ULHC (C-U)) and the (Quantify | Region of Interest (ROI) | Set ROI LRHC (C-L)) commands. Use the (View | ☐ View Region Of Interest (ROI)) to display the ROI as a rectangle from the ULHC to the LRHC (upper left-hand corner and lower right-hand corner). You can measure the integrated density of the (Quantify | Region of Interest (ROI) | Capture measurement by ROI (C-R)) command. If you set it, the (C-R) command will subtract the background computed by the area times the mean background using the (Quantify | Measure by circle | Capture background (C-B)) command.

This will compute and display background corrected data that appears in the report window as:

```
Setting ULHC (163,370) of ROI: left image
Setting LRHC (181,389) of ROI: left image
(1) plasmaH.gif (181,389) Tot(Meas-Bkgrd): 44989.309 gray
        TotMeas: 49271.000 (13.000:238.000)
        TotBkgrd: 4281.690 at (133,429) (9.000:20.000) gray
        ROI: (163:181, 370:389)
```

When grayscale calibration is added in a future version, then the measurements will be in terms of the calibration rather than grayscale.

The intent of applying image transforms is to make it easier to compare regions having similar local morphologies but with some different objects within these regions. Image warping prior to flickering is intended to spatially warp and rescale one image to the geometric "shape" of the other image so that we can compare them at the same scale. This should help make flickering of some local regions on quite different gels somewhat easier. Of the two warping transforms, affine and polynomial, the latter method handles nonlinearities better. For those cases where the gels are similar, the user may be able to get away with using the simpler (affine) transform. For demonstration purposes, if you are using the demo *plasmaH* and *plasmaL* gels, the (Landmark | Set 3 predefined landmarks for demo gels (C-Y)) and (Landmark | Set 6 predefined landmarks for demo gels (C-Z)) define 3 and 6 corresponding landmarks for these gels that may be used with the affine and polynomial warping transforms respectively.

In cases where there is a major difference in the darkness or lightness of gels, or where one gel has a dark spot and the other a very faint corresponding spot, it may be difficult to visualize the light spot. By differentially setting the flicker display-time delays, the user can concentrate on the light spot using the brief flash of the dark spot to indicate where they should look for the light spot. We have found differential-flicker to be very helpful for deciding difficult cases. Adjusting one image so that its brightness and contrast are approximately that of the other image also helps when flickering. You change the image brightness and contrast using the Shift/Drag mouse control described below.

3. Additional hints on image transforms.

Other transforms including image sharpening may be useful in cases where spots are very fuzzy, as might be the case when comparing Southern blots. When two corresponding local regions of the two images are radically different so the local morphologies are not even slightly similar (e.g., when high MW regions of gels that are run differently such as: IPG vs. non-IPG, gradient vs. nongradient SDS), then even using these transforms may not help that much.

4. Saving and restoring the Flicker state.

Flicker gives you the option of saving the current state of your session including the images your are looking at and the parameter values of the sliders, etc. To save the current state, use the (File | Save (or SaveAs) state file) command. This creates a file with a .flk file extension in the installation *FlkStartups/* folder (default *FlkStartup.flk)*. If you have used the Flicker Web site Java installer (ZeroG.com) for installing Flicker, then it lets you click on a specific *.flk* you have previously saved to restart it where you left off. While running Flicker, you can also use (File | Open state file) command to change it to another state.

5. Mouse control of images.

The following mouse and key-modified mouse operations control various actions.

Pressing the mouse in either the left or right image selects it. If flickering is active, then it will move the flicker image center for the selected image to that position. A little yellow "+" indicates the position you have selected. If the **Click to access DB** checkbox is enabled and the image has an associated active map database server associated with it, then it will request the spot identify when you click on a spot from the map database..

Dragging the mouse is similar to pressing it. However, only pressing it will invoke a clickable database. It also displays the cursor coordinates in the image title.

Control/Press will position the selected image so that the point you have clicked on will be in the center of the crosshairs. If you are near the edge of the image, it will ignore this request.

Shift/Drag activates the brightness/contrast filter with minimum brightness and contrast in the lower left hand corner.

6. Checkbox control of flickering and database access.

There are four checkboxes in the upper left part of the window that control commonly used options.

Flicker checkbox enables/disables flickering.

Click to access DB checkbox enables/disables access to a Web database server that is associated with a clickable image - if it exists for the selected image. Turning on this option will disable flickering.

Allow transforms checkbox enables/disables image transforms.

Sequential transforms checkbox enables/disables using the last image transform output as input for the next image transform (image composition) if **Allow transforms** is enabled.

7. Keyboard shortcut controls

There are several short-cut key combinations that can be use to perform operations instead of selecting the command from the pull-down menus. The notation **C-<***key***>** means to hold the Control key and then press the following <*key*>.

C-A add landmark (you must have selected both left and right image trial objects) (see Landmark menu)

C-B capture background intensity value for current image under a circular mask (see Quantify menu)

C-D delete landmark - the last landmark defined (see Landmark menu)

C-E edit selected measured spot (click on spot to select it) (see Quantify menu)

C-F toggle flickering the lower left and right images into the upper flicker window (see View menu or the ☑**Flicker (C-F)**)

C-G toggle displaying gray values in the left and right image titles as move the cursor (see View menu)

C-H show grayscale ROI histogram. Popup a histogram of the computation region ROI (see Quantify menu)

C-I define or edit selected measured spot(s) annotation ‘id’ field (see Quantify menu)

C-J toggle the spot measurement mode between list-of-spots measurement mode and the single spot trial-spot measurement mode (see Quantify menu)

C-K delete selected measured spot (click on spot to select it (see Quantify menu)

C-L define the lower right hand corner (LRHC) of the region of interest (ROI) and assign that to the computing window (see Quantify menu)

C-M measure and show intensity under a circular mask for current image. Report background-corrected value if background was defined (see C-B shortcut and Quantify menu). An alternative way to measure spots is to hold the **ALT-key** when you press the mouse to select the spot. This combines spot selection and measurement in one operation.

C-R measure and show intensity under a the computing window defined by the ROI (see commands (**C-U**) and (**C-L**)) for the current image. Report background-corrected value if circular mask background was defined (see (**C-B**) shortcut and Quantify menu)

C-T repeat the last Transform used, if one was previously performed else no-op (see Transform menu)

C-U define the upper left hand corner (ULHC) of the region of interest (ROI) and assign that to the computing window (see Quantify menu)

C-V show data-window of selected pixel in the popup report window.

C-W clear the region of interest (ROI) and computing window (see Quantify menu)

C-Y set 3 predefined landmarks for demo gels for Affine transform (see Landmark menu)

C-Z set 6 predefined landmarks for demo gels for Polywarp transform (see Landmark menu)

C-Keypad "+" increase the canvas size for all three images (see Edit menu)

C-Keypad "-" decrease the canvas size for all three images (see Edit menu)

8. Reporting the status in the popup status window.

Information is display in several places in Flicker.

a. There are two status lines in the upper left part of the main window. The output into these status lines is also appended to the Report window (c).
b. The selected image (clicking on the left or right image) changes its title to blue from black. If neither image is selected, then both titles are black.
c. A report popup window is created when Flicker is started. It may be temporarily removed by closing it. You can get it back at any time by selecting (View | Show report popup). All text output is appended to this window. The Clear button clears all text. The SaveAs button lets you save the text in the window into a local text file.

9. Sliders for defining parameters.

The following sliders are in the upper right part of the window and are used for adjusting parameters in the various image transforms.

ZoomMag (X) to zoom both left and right images from 1/10X to 10X by a transform

(3D) angle (degrees) used in the pseudo 3D transform

(3D) z-Scale (%)used in the pseudo 3D transform

(Sharpen) e-Scale(%) used in the sharpening transforms

Contrast (%) set by Shift/Drag to change the image contrast

Brightness (%) set by Shift/Drag to change the image brightness

Threshold1 (grayscale or od) is the minimum grayscale value to show pixels otherwise they are shown as whites

Threshold2 (grayscale or od) is the maximum grayscale value to show pixels otherwise they are shown as white

Meas circle (diameter in pixels) size of the measurement circle for selected image

10. Local database files.

When Flicker is installed, several tab-delimited (spreadsheet derived) *.txt* files are available in the ***DB/*** directory (located where the *Flicker.jar* file is installed). These *DB/Flk*DB.txt* files are read on startup and are used to setup the (**File | Open … image | …**) menu trees.

DB/FlkMapDB.txt - contains instances of Web-based active image maps with fields: (*MenuName, ClickableURL, ImageURL, BaseURL, DatabaseName*)

DB/FlkDemoDB.txt - contains instances of pairs of images in the local Images/ directory and contains fields:

(*SubMenuName, SubMenuEntry, ClickableURL1, ImageURL1, ClickableURL2, ImageURL2, StartupData*)

DB/FlkRecentDB.txt - contains instances of recently accessed non-demo images with fields:

(*DbMenuName, ClickableURL, ImageURL, DatabaseName, TimeStamp*)

11. Files required that are included in the download.

The following files are packaged in the distribution and installed when you install Flicker.

Flicker.jar is the Java Archive File for Flicker that is executed when you run Flicker

jai_core.jar is the core Java runtime from SUN's Java Advanced Imaging (JAI) at sun.com

jai_codec.jar is the JAI tiff file reader from SUN's Java Advanced Imaging JAI at sun.com

DB/ is a directory containing the set of tab-delimited DB files *Flk*DB.txt* read at startup

Images/ is a directory holding demo .gif, .tif, and .ppx sample files

FlkStartups/ is empty directory to put the startup *FlkStartup.flk* files

Flicker-startup.bat is a Windows .bat batch script startup file

Flicker-startup.sh is a Unix/MacOS-X command line script startup file

12. Image transform and brightness-contrast display model.

There are several display models for combinations of using image transforms, zooming, and brightness contrast filtering. Zooming is an image transform and you can de-magnify as well as magnify. These transforms and filtering are applied to the left and right windows and also are shown in the flicker window. Two checkboxes in the upper left of the main window control transforms: "Allow transform" enables/disables transforms, and "Sequential transforms" allows using the previous transform as the input to the next transform, i.e., this lets you implement image composition.

This description applies independently to the left and right images. The original image is denoted *iImg*. If you allow transforms and are also composing image transforms, you may optionally use the previous transformed output image (denoted *oImg*) as input to the next image transform. The output (either *iImg* or *oImg*) is then sent to the *output1*. Then *output1* may be optionally zoomed to output2 by being sent to the zoom transform (iff the magnification is different from 1.0X). Then *output2* may be optionally contrast-adjusted by being sent to the brightness-contrast filter (if it is active as specified by dragging the mouse in the selected window with the SHIFT-key pressed). The *output2* of the brightness-contrast filter is denoted as *bcImg*. If you have never used the zoom or brightness-contrast filtering since loading an image, then *zImg* and *bcImg* are not generated and hence not used in the displayed image. This will speed up display refresh as you navigate the windows.

a. If no transforms or brightness-contrast filtering is used on the selected image

(no transforms)
iImg → output1

b. The image may be optionally transformed from the original image (*iImg*)

(transform)
iImg → oImg → output1

c. Image transforms may be optionally composed from the original image or from the sequential composition of image transforms on the selected image

(sequential transforms)

iImg → Transform → oImg → output1

d. The image may be optionally zoomed if the magnification is not 1.0X

(zoom)
output1 → zImg → output2

or

(no zoom)
output1 → output2

e) The brightness-contrast filter may be optionally applied to the image

(B-C filter)
output2 → bcImg → display

or

(no B-C filter)
output2 → display

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Quantification of Radiolabeled Proteins in Polyacrylamide Gels

Wayne R. Springer

1. Introduction

Autoradiography is often used to detect and quantify radiolabeled proteins present after separation by polyacrylamide gel electrophoresis (PAGE) (*see* Chapter 56). The method, however, requires relatively high levels of radioactivity when weak β-emitters, such as tritium, are to be detected. In addition, lengthy exposures requiring the use of fluorescent enhancers are often required. Recent developments in detection of proteins using silver staining ***(1, 2)*** have added to the problem because of the fact that tritium emissions are quenched by the silver ***(2)***. Since for many metabolic labeling studies, tritium labeled precursors are often the only ones available, it seemed useful to develop a method that would overcome these drawbacks. The method the author developed involves the use of a cleavable crosslinking agent in the polyacrylamide gels that allows the solubilization of the protein for quantification by scintillation counting. Although developed for tritium ***(3)***, the method works well with any covalently bound label, as demonstrated here with ^{35}S. Resolution is as good as or better than autoradiography (***3*** and **Fig. 1**), turnaround time can be greatly reduced, and quantification is more easily accomplished.

2. Materials

Reagents should be of high quality, particularly the sodium dodecyl sulfate (SDS) to obtain the best resolution, glycerol to eliminate extraneous bands, and ammonium persulfate to obtain proper polymerization. Many manufacturers supply reagents designed for use in polyacrylamide gels. These should be purchased whenever practical. Unless otherwize noted, solutions may be stored indefinitely at room temperature.

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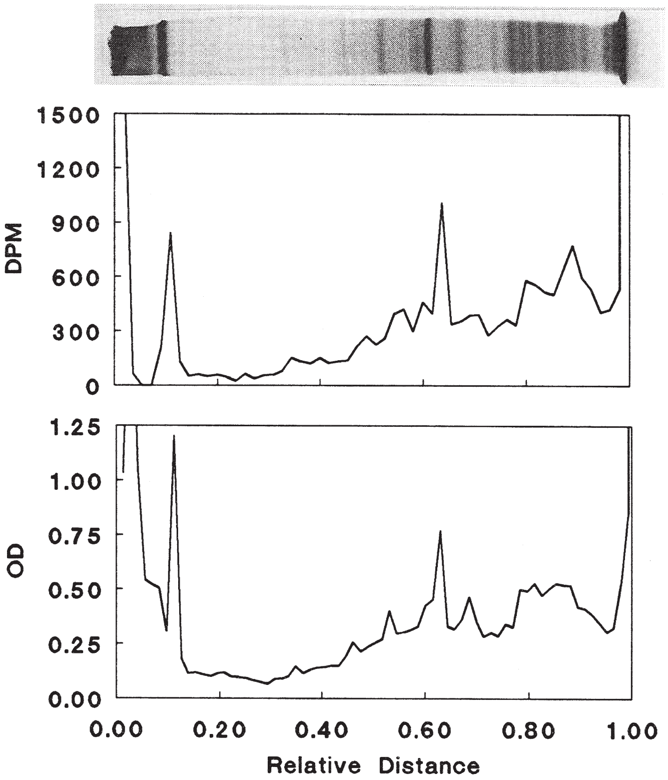

Fig. 1. Comparison of an autoradiogram, densitometry scan, and radioactivity in gel slices from a mixture of labeled proteins separated by PAGE. Identical aliquots of ^{35}S-methionine-labeled proteins from the membranes of the cellular slime mold, *D. purpureum,* were separated using the methods described in **Subheading 3.1.** One lane was fixed, soaked in Fluoro-Hance (Research Products International Corp., Mount Prospect, IL), dried, and autoradiographed (photograph). Another lane was cut from the gel, sliced into 1-mm pieces, solubilized, and counted using a Tracor Mark III liquid scintillation counter with automatic quench correction (TM Analytic, Elk Grove Village, IL) as described in **Subheading 3.3.** The resultant disintegrations per minute (DPM) were plotted vs the relative distance from the top of the running gel (**top figure**). The autoradiogram (photograph) was scanned relative to the top of the running gel using white light on a Transidyne RFT densitometer (**bottom figure**).

2.1. SDS-Polyacrylamide Gels

1. Acrylamide-DATD: Acrylamide (45 g) and *N,N′*-dialyltartardiamide (DATD 4.5 g) are dissolved in water to a final volume of 100 mL. Water should be added slowly and time allowed for the crystals to dissolve.
2. Acrylamide-*bis*: Acrylamide (45 g) and *N,N′*-methylenebis(acrylamide) (*bis*-acrylamide, 1.8 g), are dissolved as in item 1 in water to a final volume of 100 mL.

3. Tris I: 0.285 *M* Tris-HCl, pH 6.8.
4. Tris II: 1.5 *M* Tris-HCl, pH 8.8, 0.4% SDS.
5. Tris III: 0.5 *M* Tris-HCl pH 6.8, 0.4% SDS.
6. Ammonium persulfate (APS) solution: A small amount of ammonium persulfate is weighed out and water is added to make it 100 mg/mL. This should be made fresh the day of the preparation of the gel. The ammonium persulfate crystals should be stored in the refrigerator and warmed before opening.
7. *N,N,N′,N′*-tetramethylethylenediamine (TEMED) is used neat as supplied by the manufacturer.
8. 10X Running buffer: Tris base (30.2 g), glycine (144.1 g), and SDS (10.00 g) are made up to 1 L with distilled water.
9. Sample buffer: The following are mixed together and stored at 4°C: 1.5 mL 20% (w/v) SDS, 1.5 mL glycerol, 0.75 mL 2-mercaptoethanol, 0.15 mL 0.2% (w/v) Bromophenol blue, and 1.1 mL Tris I.

2.2. Silver Staining of Gels

All solutions must be made with good-quality water.

1. Solution A: methanol:acetic acid:water (50:10:40).
2. Solution B: methanol:acetic acid:water (5:7:88).
3. Solution C: 10% (v/v) glutaraldehyde.
4. 10X Silver nitrate: 1% (w/v) silver nitrate stored in brown glass bottle.
5. Developer: Just before use, 25 µL of 37% formaldehyde are added to freshly made 3% (w/v) sodium carbonate.
6. Stop bath: 2.3 *M* Citric acid (48.3 g/100 mL).

2.3. Solubilization and Counting of Gel Slices

1. Solubilizer: 2% sodium metaperiodate.
2. Scintillation fluid: Ecolume (+) (ICN Biomedical, Inc., Irvine, CA) was used to develop the method. *See* **Subheading 4**. Notes. for other scintillants.

3. Methods

The general requirements to construct, prepare, and run SDS-PAGE gels as described by Laemmli ***(4)*** are given in Chapter 11. Presented here are the recipes and solutions developed in the author's laboratory for this particular technique. For most of the methods described a preferred preparation of a standard gel can be substituted, except for the requirement of the replacement of DATD for bis at a ratio of 1:10 DATD: acrylamide in the original formulation.

3.1. SDS-Polyacrylamide Gels

Sufficient medium for one 8 × 10 × 0.75 to 1.0-cm gel can be made by combining stock solutions and various amounts of acrylamide-DATD and water to achieve the required percentage of acrylamide (*see* **Note 1**) by using the quantities listed in **Table 1**. The stacking gel is that described by Laemmli ***(4)*** and is formed by combining 0.55 mL of acrylamide-*bis* with 1.25 mL of Tris III, 3.2 mL of water, 0.015 mL of APS, and 0.005 mL of TEMED.

1. For both the running and the stacking gel, degas the solutions by applying a vacuum for approx 30 s before adding the TEMED.
2. After the addition of the TEMED, pour the gels, insert the comb in the case of the stacking gel, and overlay quickly with 0.1% SDS to provide good polymerization.
3. Prepare protein samples by dissolving two parts of the protein sample in one part of sample buffer and heating to 100°C for 2 min.
4. Run gels at 200 V constant voltage for 45 min to 1 h or until the dye front reaches the bottom of the plate.

3.2. Silver Staining

The method used is that of Morrissey ***(1)***.

1. Remove gels from the plates, and immerse in solution A for 15 min.
2. Transfer to solution B for 15 min and then solution C for 15 min, all while gently shaking.
3. At this point, the gel can be rinsed in glass-distilled water for 2 h to overnight (*see* **Note 2**).
4. After the water rinse, add fresh water and enough crystalline dithiotreitol (DTT) to make the solution 5 μg/mL.
5. After 15 min, remove the DTT, and add 0.1% silver nitrate made fresh from the 1% stock solution. Shake for 15 min.
6. Rapidly rinse the gel with a small amount of water followed by two 5–10 mL rinses with developer followed by the remainder of the developer.
7. Watch the gel carefully, and add stop bath to the gel and developer when the desired darkness of the bands is reached.
8. Store the stopped gel in water until the next step.

Table 1
Recipe for Various Percentages of SDS-PAGE Gels

	Percentage of Acrylamide		
Stock Solution	7.5%	10%	12.5%
Acrylamide-DATD	0.96 ml	1.33 ml	1.66 ml
Tris II	1.50 ml	1.50 ml	1.50 ml
Water	3.50 ml	3.13 ml	2.80 ml
APS	0.03 ml	0.03 ml	0.03 ml
TEMED	0.003 ml	0.003 ml	0.003 ml

3.3. Slicing and Counting of Gel Slices

1. Remove individual lanes from the gel for slicing by cutting with a knife or spatula.
2. Cut each lane into uniform slices, or cut identified bands in the gel (*see* **Note 3**).
3. Place each slice into a glass scintillation vial, and add 0.5 mL of 2% sodium metaperiodate solution.
4. Shake the vials for 30 min to dissolve the gel.
5. Add a 10-mL aliquot of scintillation fluid to the vial, cool the vial, and count in a refrigerated scintillation counter (*see* **Notes 4–6**).

4. Notes

4.1. SDS-Polyacrylamide Gels

1. Acrylamide-DATD gels behave quite similarly to acrylamide-bis gels, except for the fact that for a given percentage of acrylamide, the relative mobility of all proteins are reduced in the DATD gel. In other words, a DATD gel runs like a higher percentage acrylamide*bis* gel.

4.2. Silver Staining

2. The water rinse can be reduced to 1 h, if the water is changed at 10–15 min intervals. The author found in practice that the amount of DTT was not particularly critical, and routinely added the tip of a microspatula of crystals to approx 30 mL of water.

4.3. Slicing and Counting of Gel Slices

3. The method seems relatively insensitive to gel volume, as measured by changes in efficiency (***3***) over the range of 5–100 mm^3 for tritium and an even larger range for 14C or ^{35}S. Larger pieces of gel can be used if one is comparing relative amounts of label, such as in a pulse chase or other timed incorporation, but longer times and more metaperiodate may be required to dissolve the gel. Recovery of label from the gel is in the 80–90% range for proteins from 10–100 kDa (***3***). In most cases, the label will remain with the protein, but in the case of periodate-sensitive carbohydrates on glycoproteins, it may be released from the protein. This, however, does not prevent the quantification. It just does not allow the solubilized labeled protein to be recovered for other manipulations.
4. It is necessary to cool the vials in the counting chamber before counting in order to eliminate occasional chemiluminescence. The cause of this phenomenon was not explored, but one should determine whether this occurs when using other scintillants or counters.
5. **Figure 1** shows the results of a typical experiment using ^{35}S-methionine to label proteins metabolically from the cellular slime mold, Dic*tyostelium*

purpureum, and quantify them using the method described or by autoradiography. As can be seen, the resolution of the method is comparable to that of the autoradiogram (*see* **ref. *3*** for similar results using tritium). The time to process the lane by the method described here was approx 8h, whereas, the results of the autoradiogram took more than 3 d to obtain. Examination of the stained gel suggests that, if one were interested in a particular protein, it would be fairly easy to isolate the slice of gel containing that protein for quantification. This makes this method extremely useful for comparing incorporation into a single protein over time as in pulse/chase experiments ***(3)***.

6. The author has found that as little as 400dpm of tritium associated with a protein could be detected ***(3)***, which makes this method particularly useful for scarce proteins or small samples.

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Differential ProteoTope Radioactive Quantification of Protein Abundance Ratios

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1. Introduction

2D-PAGE remains a widely employed proteomics method because it provides the highest separating resolution of intact polypeptide isoforms that are amenable to subsequent characterization ***(1, 2)***. ProteoTope is a differential radioactive detection method that we developed based upon temporally separated exposures of iodinated proteins on 2D-PAGE gels ***(3, 4)***. We have also developed systems for extremely high resolution 2D-PAGE over 54 cm or longer, which were designed for optimal quantification of radioactively labeled proteins ***(5, 6)***. These methods provide synergistic improvement in data quality for obtaining quality results from limited samples, such as typical valuable clinical tissue samples ***(7–9)***.

Here we provide a comprehensive technical description of differential 2D-radioactive ProteoTope quantification using highly standardized samples from the ABRF PRG2006 study ***(10)***. Our method is a novel application of the widespread Imaging Plate radioactive detection method ***(11, 12)***. The twofold study aims for multiple ABRF study participants were to blindly identify the eight unknown standard proteins in the two sample mixtures, and to determine a best estimate of their relative amounts in each sample by multiple measurements of mixtures of the two samples at a ratio of 1:1 ***(10)***. 2D-PAGE and ProteoTope differential radioactive imaging provided excellent relative quantification data of even subtle differences between the two samples. However our inverse symmetrical replicate experimental design and precise relative quantifications revealed that the quantities of proteins in both samples were apparently altered during sample resolubilization in our laboratory. Thus this case study demonstrates

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both the excellent quantification of limited protein samples achievable by differential radioactive imaging, and the requirement for stringently optimized sample-specific handling protocols which were not developed for this one-off time-restricted experiment.

2. Materials

2.1. Samples, Resolubization, and Labeling

1. The study was performed blind on unknown standard lyophilized samples provided by the ABRF (*see* **Note 1**), and as summarized in **Table 1.**
2. Resolubilization buffer: 2% SDS, 10 m*M* Tris pH 7.4 (*see* **Note 2**).

2.2. Protein Iodination

1. Chloramine T.
2. ^{125}I specific activity >600 GBq/mg Iodide, no added carrier.
3. ^{131}I specific activity ~740 GBq/mg Iodide, no added carrier.
4. Zeba Desalting Spin Columns, 500 µL, Pierce.
5. Tributylphosphine (TBP), Fluka.
6. Bromophenol blue (BPB), Sigma.
7. Radioactive counters suitable for measuring ^{125}I & ^{131}I.
8. Lead shielding suitable to protect the operator from radiation.
9. Iodination Buffer: 8 *M* Urea, 4% CHAPS, 0.1 *M* Tris pH 7.4 (conveniently stored in 500 µL aliquots at −20°C).
10. Elution Buffer: 8 *M* urea, 2 *M* thiourea, 4% CHAPS, 4 µg/µL Tryptone (trypsinized yeast extract proteins).
11. Radiation safety equipment as specified by your authorized radiation safety officer, such as lead blocks with sufficient holes bored to hold all labeling reactions.

2.3. Isoelectric Focussing (IEF)

1. Isoelectric focusing (IEF) buffer: 7 *M* urea, 1% Triton-X-100, 10% glycerin, 2 *M* thiourea, 4% CHAPS, 1% DTT, 0,8% IPG-Buffer pH 3–10 (GE Healthcare).
2. Immobiline DryStrips (Immobilized pH gradients: IPGs), pH 3–10 linear gradient (GE Healthcare).
3. 1 % BPB solution in MilliQ water (stored in 100 µL – 200 µl aliquots at −20°C).

2.4. Equilibration

1. Equilibration buffer: 6 *M* urea, 30% glycerol, 4% SDS, 0.05 *M* Tris-HCl pH 8.8, 2% iodoacetamide. 4 m*M* TBP is added immediately prior to use.
2. Disposable rehydration/equilibration tray with Lid, 24 cm, Biorad.

2.5. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 30% Acrylamide/ 0,8% Bis-acrylamide-stock solution (C. Roth, Catalogue Nr 3029.1).
2. 1.5 *M* Tris-HCl, pH 8.8. (May be autoclaved for long term storage).

Table 1
Nominal sample description

	1*	2*	3*	4*	5*	6*	7*	8	9	10	11
	MW	pI	Mixture A	Mixture B	Mixture A	Mixture B	Nominal Ratio	Mixture A	Mixture B	Mixture A	Mixture B
			µg	µg	pmol/vial	pmol/vial		pmol/‘5 µg’	pmol/‘5 µg’	pmol/gel	pmol/gel
Beta Casein	23.4	5.0	14	3.5	598	150	4:1	37.4	9.4	4.20	1.05
Catalase	57.5	6.4	17.2	3.4	299	59	5:1	18.7	3.7	2.10	0.41
Glycogen Phosphorylase	97.1	6.8	0.3	22.8	3	235	1:76	0.2	14.7	0.02	1.65
Carbonic Anhydrase	28.9	6.6	0.8	2.6	28	90	1:3	1.8	5.6	0.20	0.63
Horseradish Peroxidase	35.6	5.6	10.6	10.6	298	298	1:1	18.6	18.6	2.10	2.10
Ribonuclease A	13.5	5.8	4	4	296	296	1:1	18.5	18.5	2.08	2.08
Bovine Serum Albumin	66.3	5.6	12.9	12.9	195	195	1:1	12.2	12.2	1.37	1.37
Lactoperoxidase	80.4	8.4	24.1	24.1	300	300	1:1	18.8	18.8	2.11	2.11
			83.9	83.9				126.1	101.4		

*SSNominal values from the ABRF *(**10**)*.

Columns 1–7 (asterisked) were taken from information supplied by the ABRF after the results were announced (***10***). Columns 8 and 9 show the nominal amount of each protein present in the 5 µg radiolabeling reactions, and columns 10 and 11 show the nominal amounts of each protein loaded per 2D PAGE gel, assuming 100% yields.

3. 10% sodium dodecyl sulfate (SDS) stock solution in MilliQ water.
4. 10% ammonium persulfate (APS) solution. (Mix fresh each time by weighing crystalline APS and adding 9 times that weight of MilliQ water).

2.6. Gel drying & lamination

1. 4 × vacuum Gel Dryers (Speed Gel SG210D, ThermoSavant).
2. Plastic kitchen foil.
3. 3 m*M* blotting paper.
4. Laminating Pouch Film large enough for your gels (for ISO-DALT gel we used 216 × 303 mm, 80 microns; Bürobedarf Keller, Marktredwitz, Germany)
5. A laminating device and foils large enough for your gels (Filux LM1250HC DIN A3, Topversand24 GbR, 26135 Oldenburg, Germany)
6. Radiation safety equipment as approved by your radiation safety officer for the process.

2.7. Image Acquisition

1. 20× Fuji Imaging Plates (IPs) for 21–22 h (BAS-MS 2325; raytest GmbH, Straubenhard, Germany).
2. 20 × IP imaging cassettes for IP exposure (raytest GmbH, Straubenhard, Germany).
3. Fuji FLA-3000 R Bioimager and accompanying computer (raytest GmbH, Straubenhard, Germany).
4. IP erasing capacity for 20 IP plates (we use an own-made apparatus).
5. Suction device to remove IPs from the cassettes (optional) (raytest GmbH, Straubenhard, Germany).
6. Copper and lead lined cabinet (safe) to expose the IPs (optional) (raytest GmbH, Straubenhard, Germany).

2.8. Image Deconvolution

1. A computer program capable of image deconvolution. We use the own-developed “select_deconv_v1.1.pl” program (ProteoSys AG)

3. Methods

3.1. Sample Resolubilization and Protein Labeling

1. The desiccated samples were dissolved directly into 40 µL of 10 mM Tris, 2% SDS, pH7.4 with 5 min shaking at 95°C (*see* **Note 2**). Protein content was assumed to be 80 µg/40 µl based upon the ABRF sample description.

3.2. Protein iodination

1. Into eight reaction tubes place 60 µL Iodination Buffer.
2. Design your own experimental replicates as appropriate. For the described ABRF study experiment: Transfer 2.5 µL (5 µg) of the redissolved sample A into four of the reaction tubes, and transfer 2.5 µL of sample B into the other four reaction tubes. This establishes inverse duplicates samples for eight iodination reactions:

^{125}I-A1, ^{131}I-A1, ^{125}I-A2, ^{131}I-A2, ^{125}I-B1, ^{131}I-B1, ^{125}I-B2 and ^{131}I-B2 (*see* **Notes 3** and **4**).

3. Transfer 3,7 MBq of ^{125}I to each tube of sample A or B that is to be labeled with ^{125}I from the previous step (^{125}I-A1, ^{125}I-A2, ^{125}I-B1, and ^{125}I-B2). Then transfer 3,7 MBq of ^{131}I to the other tubes (^{131}I-A1, ^{131}I-A2, ^{131}I-B1, and ^{131}I-B2). (*see* **Note 5**).
4. Start the reaction by adding 3.5 µL of 100 m*M* Chloramine T (20X concentrated stock solutions: reaction conditions 5 m*M*).
5. Reaction time is 5 min at RT.
6. The reaction is stopped by adding 2 µL of 1 M TBP in 2-propanol (*see* **Note 6**).
7. The labeled proteins are purified by Spin columns (*see* **Note 3**).
8. Remove the bottom seal of the column and loosen (do not remove) the cap.
9. Place the column in a 1.5–2.0 mL microcentrifuge collection tube.
10. Centrifuge at 1,500 × *g* for 1 min to remove storage solution.
11. Place a mark on the side of the column where the compacted resin is slanted upward. Place the column in the microcentrifuge with the mark facing outward in all subsequent centrifugation steps.
12. Add 300 µL of elution buffer on top of the resin bed. Centrifuge at 1,500 × *g* for 1 min to remove buffer.
13. Repeat the previous step three additional times, discarding buffer from the collection tube.
14. Place the column in a new collection tube, remove the cap, and apply the sample to the top of the compacted resin bed. Load sample directly to the centre of the resin bed. Carefully touch the pipette tip to the resin to expel all sample.
15. Centrifuge at 1,500 × *g* for 2 min to collect the sample.
16. Discard the desalting column after use.
17. Measure radioactivity of the resulting protein solutions.
18. Calculate the amount of iodine incorporated into proteins (*see* **Notes 7** and **8**) to assess the labeling yield (*see* **Note 9**).
19. All procedures should be approved by your radiation safety officer.

3.3. Isoelectric Focusing (IEF)

The methods of 2D-PAGE are so well established that these are described only in general reference to the steps employed. It is assumed that each laboratory has existing 2D-PAGE methods and equipment available and operating. For general methodical 2D-PAGE references see ***(13–16)*** or reference ***(17)*** from the Methods in Molecular Biology series. Samples were analyzed by 2D-PAGE according to the regime of **Fig. 1A** (*see* **Note 4**).

1. Prepare 20 Eppendorf tubes and 20 loading mixtures as follow:
2. Add 200 µL IEF buffer to each vessel.
3. Add 90 kBq of the appropriate ^{125}I-labelled protein and ^{131}I-labelled to each well according to the scheme of **Table 2.** Adjust the volume with IEF buffer up to 350 µL. Add 2.8 µL of IPG buffer pH 3–10 linear. Be sure to use a new disposable tip each time.

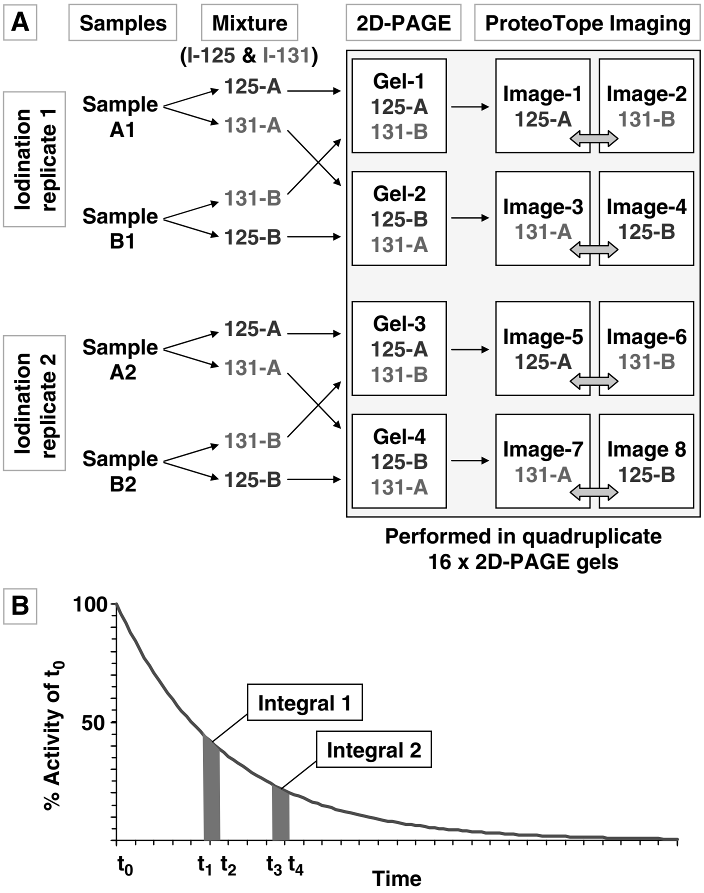

A
Samples
Mixture
(I-125 & I-131)
2D-PAGE
ProteoTope Imaging
Iodination replicate 1
Sample A1
125-A
131-A
Sample B1
131-B
125-B
Iodination replicate 2
Sample A2
125-A
131-A
Sample B2
131-B
125-B
Gel-1 125-A 131-B
Gel-2 125-B 131-A
Gel-3 125-A 131-B
Gel-4 125-B 131-A
Image-1 125-A
Image-2 131-B
Image-3 131-A
Image-4 125-B
Image-5 125-A
Image-6 131-B
Image-7 131-A
Image 8 125-B
Performed in quadruplicate
16 x 2D-PAGE gels
B
100
50
% Activity of t_0
Integral 1
Integral 2
t_0
t_1 t_2
t_3 t_4
Time

Fig. 1. Schematic depiction of ProteoTope analysis in this study. (**A**) Experimental ProteoTope Inverse Replicate and Tracer Gel Design employed for quantitative analytical multiplex. In this study the design for boxed gels 1–4 was performed in quadruplicate to generate 16 × 2D-PAGE gels, pH 3–10 linear. Each sample is separately iodinated in duplicate with each of two radioactive iodine isotopes: ^{125}I (dark) and ^{131}I (light). The radioactively labeled proteins were mixed together and coelectrophoresed on inversely labeled replicate 2D-PAGE gels as shown. The individual signals from each radioisotope were measured by ProteoTope and mutually calibrated to give equal BSA intensities in each channel of a respective gel. Double headed arrows represent multiplex image generation. The experimental design provides 16 independent estimates of the abundance ratio of proteins in particular spots between the samples, and includes all variables introduced by sample handling and electrophoretic conditions. (**B**) A schematic depiction of the integrals between t1 and t2, and t3 and t4 (being time intervals after t0), used to calculate decay factors that are provided in **Table 2**.

4. Prepare 20 IEF Rehydration tubes. The tubes should be made of 5 mL disposable pipettes (606107, Greiner Bio-One GmbH). Cut each pipette at the ends to obtain 22 cm long tubes.
5. Place into each tube Immobiline dry strip 18 cm, pH 3–10, gel side down.
6. Put the cup (Caps, 12504, Moss Plastic Parts) at one end of each tube. Label the tubes as Loading mixture.
7. Apply the Loading mixtures into appropriate strip. Carefully pipette the mixture between the gel side of the strip and the wall of the tube. Avoid introducing bubbles (*see* **Fig. 2**). Close the tube with the cap.
8. Incubate horizontally (gel side down) overnight at RT.
9. Rinse the rehydrated IPG strips with water, blot lightly onto filter paper soaked with water and place them gel side up in the strip aligner tray of a Multiphor IEF device.
10. Cut paper electrode strips (ca 15–20 mm wide and approx. 130 mm long for the IPG-Phor from Macherey-Nagel Filterpaper MN 440. Soak the paper strips with water and blot excess water with clean blotting paper, then place the wet paper electrode strips over the ends of IPG strips so that the electrode strip overlaps onto about 5 mm of the IPG gel.
11. Install electrodes so that they press down roughly in the middle of the paper electrode strips.
12. Switch on the water cooling system to maintain constant temperature at 25°C.
13. With the power supply limited to 2 mA and 5 W, electrophorese the samples for 1 h at 150 V, 1 h at 300 V, 0.5 h at 600 V, 0.5 h 1500 V, 0.5 h at 2000 V, 1 h at 3500 V, 10 h at 6000 V (*see* **Note 5**).
14. All procedures should be approved by your radiation safety officer.

3.4. Equilibration

1. After the completion of IEF, incubate each individual IPG for 30 min, in 5 mL Equilibration Buffer, while gently shaking. Use disposable equilibration trays (*see* **Note 3**).
2. All procedures should be approved by your radiation safety officer.

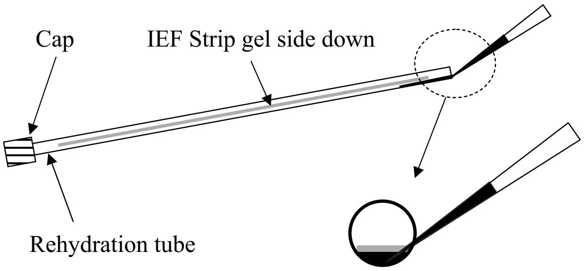


Fig. 2. Loading of rehydration mixture.

3.5. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 20 × 12% acrylamide SDS gels were electrophoresed in one ISO-DALT chamber, loading two gels per ISO-DALT slot, for 3 h at 50 V, and 110 V overnight with temperature regulated to 20°C.
2. All procedures should be approved by your radiation safety officer.

3.6. Gel Drying and Llamination

1. Separate the 2D-PAGE gels from one of the glass plates, systematically removing the glass plate from the same side of all gels. The aim is to process all gels in the same orientation so that all resulting gel images will have the same orientation. If some gels are processed in the wrong orientation the images can still be later manually rotated or mirrored by computer.
2. Place a piece of blotting paper on top of the gel, conveniently precut to about 23 × 30 cm (*see* **Notes 3** and **10**).
3. Invert the glass plate and paper, so that the paper is underneath the glass and gel, then gently remove the glass plate.
4. Place plastic kitchen foil over the gel, fully covering all potential sources of radioactivity.
5. Place the gel, paper down and plastic up, on top of two sheets of blotting paper on a gel dryer. (Up to four ISO-DALT gels can fit onto one gel dryer.)
6. Dry the gels at 80°C under vacuum for at least 45 min up to 90 min (*see* **Note 11**) on the Savant gel dryer (*see* **Note 5**).
7. Place the gels, dried onto one sheet of paper, into 0.08 mm plastic protective layer laminating foils, paying careful attention that the orientation of all gels is the same. Obviously, avoid contaminating the outside of the plastic! These will later be in direct contact with the IPs and you cannot afford a contamination of the IPs with ^{125}I, which has a half-life of 60 days.
8. Seal the gel in the laminating foil using the lamination device. The seal should ideally be perfect and airtight. At this stage the gels are ready for imaging.
9. All procedures should be approved by your radiation safety officer.

3.7. Image Acquisition

1. Erase 20 IP plates (*see* **Note 12**).
2. Place the gels into an IP exposure cassette, paper side down and gel side up, once more paying attention that the orientation of all gels is identical (*see* **Note 13**).
3. Gently place a freshly pre-erased IP into the cassette, white/phosphor side down and contacting the upturned gel surface of the laminated gel, also paying attention that the edges are flush with the edges of the exposure cassette in one corner. Be careful not to bend the IP or touch the white surface with your fingers (*see* **Note 12**).
4. Close the cassette, noting the exact time for each different IP to give the individual t1 values analogous to those from **Table 2** (*see* **Note 14**).
5. Expose for 21–22 h (*see* **Note 15**).

6. After the exposure is complete, open the IP exposure cassette under conditions of low light (*see* **Note 16**). Record the exact time the exposure is terminated to give t2 from **Table 2.**
7. Carefully remove the exposed IP from the cassette containing the laminated gel and transfer the IP to the imaging device for scanning (*see* **Note 17**). Pay attention to the consistent and exact orientation of all IPs with respect to the IP stage (*see* **Note 18**).
8. Place the IP stage with IP into the Imager, which has been switched on for sometime beforehand to warm up the lasers (*see* **Note 19**).
9. Scan on a Fuji FLA-3000 R Bioimager at 0.2 mm resolution using the IP filter selection and grid setting height = 1125, width = 1250 of the BASReader software (Version 3.01) (*see* **Note 20**).
10. When the images are acquired the IP may be removed. Remove the IP stage from the imager and take the IP off it by sticking your gloved finger through the hole in the stage taking care once more not to touch the white surface with your bare fingers or to bend the IP.
11. The IP may then be erased.
12. Acquisition of the first scan images is complete.
13. After waiting at least two weeks (*see* **Note 21**) to permit differential decay of the ^{125}I and ^{131}I (half lives of 59.41 days for ^{125}I and 8.04 days for ^{131}I) on the labeled proteins, repeat this entire exposure and imaging process to generate the second scan images (*see* **Note 22**). Remember to record t3 (commencement of the second exposure) and t4 (termination of the second exposure) from **Table 2** for each individual exposure.

3.8. Image deconvolution

1. Expositions in this study were commenced on 10 November 2005 and 1 December 2005 according to the regime of **Table 2.**
2. The signal distribution of ^{125}I and ^{131}I on the gel images is deconvoluted by solving simultaneous equations with a computer algorithm to generate images of the two individual isotopes on each gel (*see* **Notes 23** and **24**). An example set of images for gel 1 from **Table 2** is depicted in **Fig. 3.**

3.9. Image processing and spot detection

Quantitative image processing was performed with the PIC/GREG software package (Fraunhofer-Institut für Angewandte Informationstechnik, Sankt Augustin, see http://www.fit.fraunhofer.de/projekte/greg/index_en.xml). There are multiple commercially available software products suitable for 2D-PAGE image processing. The signal intensities of deconvoluted images for each isotopic channel were calibrated to produce a best-fit 1:1 ratio for albumin spots for each gel according to sample information provided by the ABRF ***(10)***. Therefore final abundance ratio estimates were relative to the assumed albumin ratio. (Note that this is not our standard normalization method. We usually normalize

Table 2
2D-PAGE gels performed in this study, and deconvolution parameters

								I-125			I-131		
Gel-No.	I-125	I-131	cold protein	t1[a]	t2	t3	t4	Integral 1	Integral 2	DF 125	Integral 1	Integral 2	DF 131
1	A1	B1		4.90	5.75	25.82	26.75	0.8038	0.6847	**1.174**	0.5406	0.0965	**5.601**
2	A1	B1		4.90	5.76	25.82	26.76	0.8141	0.6907	**1.179**	0.5473	0.0973	**5.624**
3	B1	A1		4.90	5.77	25.82	26.77	0.8209	0.6968	**1.178**	0.5518	0.0982	**5.621**
4	B1	A1		4.90	5.78	25.82	26.78	0.8271	0.7038	**1.175**	0.5558	0.0991	**5.608**
5	A2	B2		4.90	5.78	25.82	26.78	0.8333	0.7086	**1.176**	0.5599	0.0998	**5.612**
6	A2	B2		4.90	5.80	25.82	26.79	0.8460	0.7136	**1.186**	0.5681	0.1004	**5.656**
7	B2	A2		4.90	5.82	25.82	26.80	0.8684	0.7189	**1.208**	0.5826	0.1012	**5.759**
8	B2	A2		4.90	5.86	25.82	26.81	0.9089	0.7238	**1.256**	0.6088	0.1018	**5.979**
9	A1	B1		4.90	5.87	25.82	26.81	0.9155	0.7286	**1.257**	0.6131	0.1025	**5.983**
10	A1	B1		4.90	5.88	25.82	26.82	0.9228	0.7336	**1.258**	0.6178	0.1032	**5.989**
11	B1	A1		4.98	5.89	28.88	29.77	0.8467	0.6363	**1.331**	0.5648	0.0715	**7.897**
12	B1	A1		4.98	5.90	28.88	29.78	0.8579	0.6433	**1.334**	0.5720	0.0723	**7.914**
13	A2	B2		4.98	5.92	28.88	29.79	0.8731	0.6484	**1.346**	0.5817	0.0728	**7.987**
14	A2	B2		4.98	5.93	28.88	29.80	0.8838	0.6534	**1.352**	0.5886	0.0734	**8.022**
15	B2	A2		4.98	5.94	28.88	29.80	0.8947	0.6583	**1.359**	0.5957	0.0739	**8.060**
16	B2	A2		4.98	5.95	28.88	29.81	0.9014	0.6634	**1.359**	0.5999	0.0745	**8.058**
17	A1	A1		4.98	5.96	28.88	29.82	0.9147	0.6692	**1.367**	0.6085	0.0751	**8.104**
18	A2	A2		4.98	5.97	28.88	29.83	0.9215	0.6739	**1.367**	0.6129	0.0756	**8.107**
19	B1	B1		4.98	5.97	28.88	29.85	0.9280	0.6880	**1.349**	0.6170	0.0771	**8.001**
20	B2	B2		4.98	5.98	28.88	29.85	0.9344	0.6931	**1.348**	0.6211	0.0777	**7.997**

21	A1		A1
22	A1		A1
23	B1		B1
24	B1		B1

Text tabular version

Gel-No.	I-125	I-131	cold protein	t1[a]	t2	t3	t4	Integral 1	Integral 2	**DF 125**	Integral 1	Integral 2	**DF 131**
1	A1	B1		4,90	5,75	25,82	26,75	0,8038	0,6847	**1,174**	0,5406	0,0965	**5,601**
2	A1	B1		4,90	5,76	25,82	26,76	0,8141	0,6907	**1,179**	0,5473	0,0973	**5,624**
3	B1	A1		4,90	5,77	25,82	26,77	0,8209	0,6968	**1,178**	0,5518	0,0982	**5,621**
4	B1	A1		4,90	5,78	25,82	26,78	0,8271	0,7038	**1,175**	0,5558	0,0991	**5,608**
5	A2	B2		4,90	5,78	25,82	26,78	0,8333	0,7086	**1,176**	0,5599	0,0998	**5,612**
6	A2	B2		4,90	5,80	25,82	26,79	0,8460	0,7136	**1,186**	0,5681	0,1004	**5,656**

								I-125			I-131		
Gel-No.	I-125	I-131	cold protein	t1[a]	t2	t3	t4	Integral 1	Integral 2	DF 125	Integral 1	Integral 2	DF 131
8	B2	A2		4,90	5,86	25,82	26,81	0,9089	0,7238	**1,256**	0,6088	0,1018	**5,979**
9	A1	B1		4,90	5,87	25,82	26,81	0,9155	0,7286	**1,257**	0,6131	0,1025	**5,983**
10	A1	B1		4,90	5,88	25,82	26,82	0,9228	0,7336	**1,258**	0,6178	0,1032	**5,989**
11	B1	A1		4,98	5,89	28,88	29,77	0,8467	0,6363	**1,331**	0,5648	0,0715	**7,897**
12	B1	A1		4,98	5,90	28,88	29,78	0,8579	0,6433	**1,334**	0,5720	0,0723	**7,914**
13	A2	B2		4,98	5,92	28,88	29,79	0,8731	0,6484	**1,346**	0,5817	0,0728	**7,987**
14	A2	B2		4,98	5,93	28,88	29,80	0,8838	0,6534	**1,352**	0,5886	0,0734	**8,022**
15	B2	A2		4,98	5,94	28,88	29,80	0,8947	0,6583	**1,359**	0,5957	0,0739	**8,060**
16	B2	A2		4,98	5,95	28,88	29,81	0,9014	0,6634	**1,359**	0,5999	0,0745	**8,058**
17	A1	A1		4,98	5,96	28,88	29,82	0,9147	0,6692	**1,367**	0,6085	0,0751	**8,104**

(continued)

Table 2 (continued)

Gel-No.	I-125	I-131	cold protein	t1[a]	t2	t3	t4	I-125			I-131		
								Integral 1	Integral 2	DF 125	Integral 1	Integral 2	DF 131
18	A2	A2		4,98	5,97	28,88	29,83	0,9215	0,6739	**1,367**	0,6129	0,0756	**8,107**
19	B1	B1		4,98	5,97	28,88	29,85	0,9280	0,6880	**1,349**	0,6170	0,0771	**8,001**
20	B2	B2		4,98	5,98	28,88	29,85	0,9344	0,6931	**1,348**	0,6211	0,0777	**7,997**
21	A1		A1										
22	A1		A1										
23	B1		B1										
24	B1		B1										

[a] The actual time point of t1 for Gel no. 1 was 10 Nov. 2005, 14:16 (hh.mm).

Samples A1, A2, B1, and B2 correspond to replicates of Sample A and Sample B. Gels 1–16 were used to quantify the differential abundances of proteins between samples A and B. Gels 17–20 were labeling controls, and gels 21–24 were preparative gels, loaded with 10 µg of the indicated non-radioactively labeled proteins (cold protein) per gel to be used for mass spectrometric protein identification (not described here). t1 and t2 are given in days after the calibration date of ^{125}I. These represent the start and finish of the first exposure, and are used to calculate Integral 1 for each isotope ^{125}I and ^{131}I, according to the theoretical fraction of radioactive decay between t1 and t2 and as schematically depicted in Fig. 1B. Integral 2 is similarly calculated from t3 and t4. Integrals 1 and 2 are then used to calculate the decay factors (DF) for deconvolution for each isotope as indicated. The decay factors shown correspond to 1/M and 1/N from equation 2 (see Note 23).

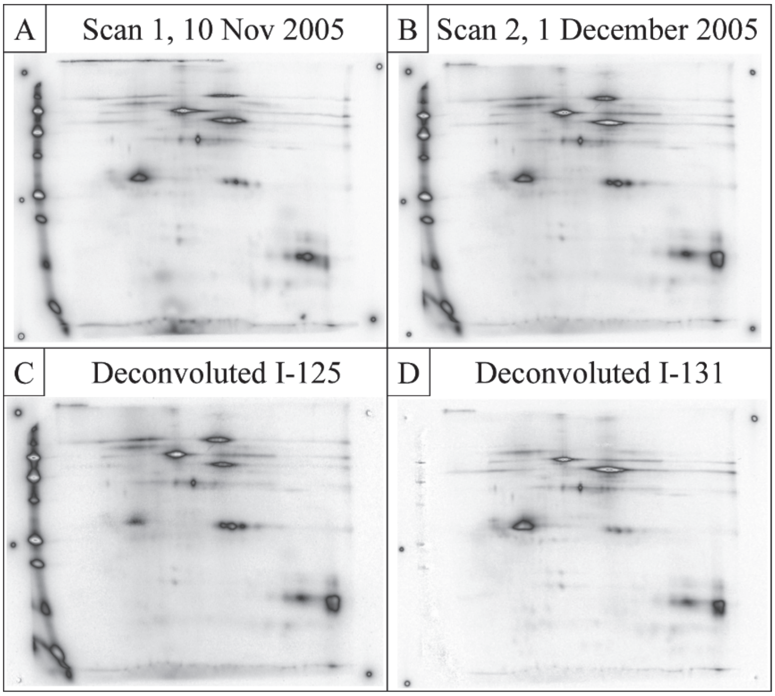
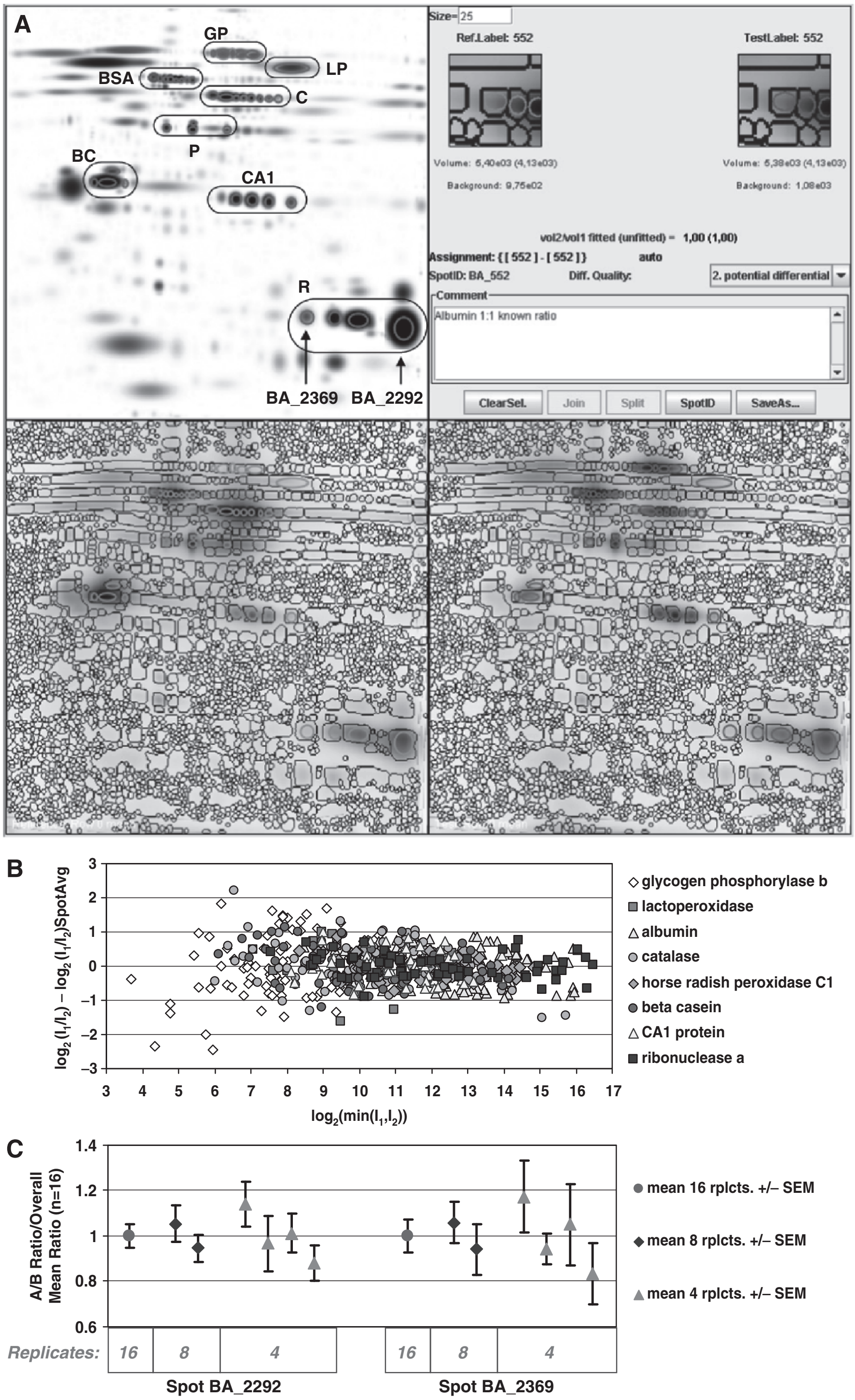


Fig. 3. Deconvolution of gel 1 from **Table 2.** **(A)** The signal obtained from the initial exposure, made for 21 h starting on day 1. **(B)** The signal obtained from the second exposure, made for 22 h starting on day 21. **(C)** The deconvoluted signal from the ^{125}I-labeled sample. **(D)** The deconvoluted signal from the ^{131}I-labeled sample. Deconvolution was performed according to **Eqs. 1** and **2** (*see* **Note 23**) using parameters shown in **Table 2**. Our deconvolution process produces non-linearly scaled TIFF images from the floating point FLA3000 data files.

the total signal volumes for both isotopic channels to each other.) The composite average gel images for sample A and sample B from all 16 gels are shown in **Fig. 4,** as are data depicting the reproducibility of data acquisition for the replicates of different 2D spots for the test proteins.

3.10. Statistical analysis

Quantitative values from PIC/GREG were exported in a spreadsheet and statistics were performed using Excel (Microsoft). The mean values and standard errors are modeled from the $\log_2(I_{sample2}/I_{sample1})$ intensity ratio values, taking advantage of the gel replicates. *P*-values are calculated using a t-test based on the null hypothesis of identical populations unless otherwise specified. The overall result for all spots used for quantitative analysis is shown in **Fig. 6,**



Size= 25

Ref.Label: 552

TestLabel: 552

Volume: 5,40e03 (4,13e03)

Background: 9,75e02

Volume: 5,38e03 (4,13e03)

Background: 1,08e03

vol2/vol1 fitted (unfitted) = 1,00 (1,00)

Assignment: {[552] - [552]} auto

SpotID: BA_552

Diff. Quality: 2. potential differential

Comment

Albumin 1:1 known ratio

and the comparison of ProteoTope-estimated abundance ratios with those given by the ABRF as nominally correct is shown in Fig. 6 (*see* **Notes 25** and **26**).

4. Notes

1. A technical description of the samples has been published ***(10)***. The samples (one vial of Sample A, one vial of Sample B) from the ABRF arrived in our premises on 31 October 2005. The deadline for submitting results was no later than 9 December 2005. ABRF-PRG2006 was composed of two mixtures, labeled A and B, that contained eight major unknown proteins each of various unknown amounts according to the sample description. The total amount of unknown protein in each sample vial was given as approximately 80 µg. Individual proteins were present on average at

◄

Fig. 4. Master gel and spot quantification. **(A)** A screen shot showing the spot detection made with the PIC/GREG software using the 32 images from all 16 measured 2D gels. The upper left panel shows a master synthetic differential image, with the positions of spots used to estimate the abundance ratios of the eight test proteins in **Fig. 5.** The proteins identified (with SwissProt Accession and number of spots quantified per protein in this study) in order of increasing SDS-PAGE mobility were: GP = Glycogen Phosphorylase B (P00489, $n = 5$); LP = Lactoperoxidase (P80025, $n = 1$); BSA = Bovine Serum Albumin (P02769, $n = 8$); C = Catalase (P00432, $n = 8$); P = Horse Radish Peroxidase (P00433, $n = 4$); BC = Beta Casein (P02666, $n = 4$); CA1 = Carbonic Anhydrase I (P00915, $n = 4$); R = Ribonulclease A (P61823, $n = 4$). Spots were selected for quantification only after unambiguous mass spectrometric identification. All identifications matched the identities supplied by the ABRF (***10***). BA_2369 and BA_2292 are the spots in C below. The lower two panels show the spot segmentation performed by PIC/GREG on the averaged gel images for Sample A **(lower left panel)** and Sample B **(lower right panel)**. These averaged images are synthetically constructed using the 16 warped images generated with both isotopes of iodine for each sample. The upper right panel shows a user interface containing spot parameters for the selected most acidic BSA spot. **(B)** Spot intensity ratios over minimum spot intensity. Logarithms to base 2 of the ratios of spot intensities under conditions A over B minus $\log_2$ of the average intensity ratio of the respective spot $\log_2(I_1/I_2)$ - $\log_2(I_1/I_2)$SpotAvg are displayed for each gel replicate. Data points are plotted as a function of the $\log_2$ of the minimum spot intensity for either Sample **A** or **B** $\log_2(\min(I_1,I_2))$. Symbols indicate the proteins that were identified in the respective spots. The diagram demonstrates that the measurement error in replicate spot quantifications becomes smaller as signal intensities increase. **(C)** Dependency of error on replicate number. The estimated mean spot volumes (with standard errors of means) for the most intense (Spot BA_2292) and least intense (Spot BA_2369) spots for Ribonuclease A are plotted using estimates made from four sets of $n = 4$, two sets of $n = 8$, or one set of $n = 16$ replicates (rplcts), plotted as fold overall mean ratio for the $n = 16$ set (A/B Ratio/Overall Mean Ratio ($n = 16$)). All estimated mean abundance ratios for both spots were within 20% of the mean obtained for n=16 gels. Gel grouping was numerical from **Table 2** (i.e., 1–4, 5–8, 9–12, 13–16, 1–8, 9–16, 1–16).

300 pmol, ranging from approximately 3 to 600 pmol, with ratios of proteins between mixtures A and B that could vary by up to 1:100. Some of the information as presented by the ABRF PRG2006 committee after completion of the study on the abundance of sample proteins is summarized in columns 1–7 of **Table 1.** The overall comparative results of the study for all participants using all experimental methods have been published, where ProteoTope is referred to as "2D radioactivity" ***(10)***.

2. We were the only participant in this study to resolubilize the desiccated protein pellet in an SDS-buffer. This was a one-off real life experiment without method optimization. This method apparently did not resolubilize all desiccated proteins with equal efficiency. We would recommend lysing frozen cryogenically sliced tissue samples into a boiling SDS or a urea-based buffer. For desiccated protein samples a urea buffer is apparently more successful.
3. Be sure to label all reaction vessels and other objects beforehand to avoid unnecessary exposure to radioactivity.
4. The inverse replicate experimental design described here systematically controls for errors that may be introduced by radiolabeling or electrophoretic laboratory steps. Proteins were prepared for 2D-PAGE as described ***(8, 10–12)*** without cysteine alkylation prior to radiolabeling due to schedule constraint and the associated inability to optimize conditions.
5. 1 mm lead shielding can be placed over apparatus to avoid exposure from ^{125}I, however note that gamma radiation from ^{131}I is not effectively impeded by such thin lead sheeting, and so the best protection from radiation exposure is substantial shielding such as a lead brick wall, preferably combined with sufficient physical distance (e.g., leave the room when you are not working). Your radiation safety officer should approve the protocol that you implement.
6. The concentrated form of TBP is explosive in the presence of oxygen, however a 200 mm stock solution is not explosive and can be stored at 4 degrees under inert gas. The stock solution is prepared by diluting the concentrated form (3.93 *M*) in solvent under an inert gas. TBP also is volatile, toxic, and unpleasant smelling ***(18)***, and should be handled in a fume hood.
7. Briefly, iodination reactions with either ^{125}I or ^{131}I were conducted under identical chemical iodine concentrations, using 3.7 MBq of each isotope (^{125}I specific activity >600 GBq/mg Iodide, no added carrier; ^{131}I specific activity ~ 740 GBq/mg Iodide) per 5 µg sample aliquot in a reaction volume of 60 µL by the chloramine T method as described ***(19)***, taking all appropriate radiation safety precautions. Iodine incorporation into proteins was determined to be approximately 800 kBq per labeling reaction, with the yield of iodine incorporated into proteins ranging from 18.6% to 21.1%.

Table 3
Theoretical level of labeling of an 'ideal average' 40 kDa protein and of average tyrosine residues at maximum theoretical specific activity of each isotope

	1 fmol/kBq	2 fmol/800 kBq	3 mol 40 kDa/mol Iodine	4 mol Tyr/mol Iodine
I-125	12,39	9912	13	151
I-131	1,67	1336	94	1123

Column 1 shows the approximate maximum theoretical specific radioactivity per Becquerel of the iodine isotopes used to label proteins, and column two shows the corresponding approximate number of femtomoles of radioactive iodine atoms incorporated into proteins in ideal 5 μg radiolabeling reactions with yield of 800 kBq. Column 3 shows the ratio moles of an "ideal average" 40 kDa protein per mole of incorporated iodine. Column 4 shows the number moles of tyrosine per radioiodinated tyrosine residue in the "ideal sample" (assuming an "average amino acid residue of 110 Da" and a tyrosine amino acid frequency of 0.033 for the "ideal average" 40 kDa protein). In our case, the specific activities of the ^{125}I and ^{131}I isotopes employed were approximately equal, indicating that ^{131}I had decayed through between two and three half-lives at the time of calibration (16% max. theoretical specific activity: a value which decreases by 50% each 8 days), and therefore the chemical labeling stoichiometry achieved for ^{131}I in this experiment was within approximately 23% of that given for ^{125}I above (i.e. 186 mol Tyr/mole iodine corresponding to column 4). Similarly, the specific activity of the ^{125}I employed was within 7% of its maximum theoretical value, and so the labeling stoichiometry of ^{125}I should have been within 7% of that given for ^{125}I in the table (i.e. >140 mol Tyr/mole iodine).

8. To elaborate upon the degree of labeling achieved in this experiment, it may be useful to consider the state of labeling of a population of "ideal average 40 kDa proteins" of random sequence with iodine isotopes of maximum theoretical specific activity as depicted in **Table 3.** Each 5 μg labeling reaction would contain 125 pmol of such "average 40 kDa proteins," which accords well with the nominal sum tallies of 126 pmol and 101 pmol for the experimental sample proteins of columns 8 and 9 from **Table 1**. Assuming that all the iodine present in the yield of radioactive protein was present as labeled tyrosine (an approximately correct yet ideal assumption which ignores nonspecific side reactions and noncovalent affinity of iodine to the protein mixture), approximately every thirteenth "ideal 40 kDa protein molecule" would be iodinated with ^{125}I and each ninetieth 40 kDa protein would be iodinated with ^{131}I under these reaction conditions. This would correspond to approximately every one hundred and fiftieth tyrosine in the mixture being labeled with ^{125}I or less than every thousandth tyrosine being labeled with ^{131}I. Our actual chemical labeling conditions were similar for both isotopes because of

relatively higher proportion of ^{127}I (nonradioactive stable isotope) in the ^{131}I preparation, as described in the legend to **Table 3.** Although these values are more qualitative than quantitative, they illustrate that the labeling conditions employed are conservative in order to reduce the possibility of generating double labeled proteins and corresponding protein chains.

9. As is evident from **Table 3** and **Note 8,** we labeled only a small fraction of the protein molecules present. This is performed to provide a margin of safety for the labeling conditions. To understand this, it is necessary to consider the production of the isotopes in some detail. The iodine is usually produced by irradiating a target of Te in a cyclotron. For instance, in one method to produce ^{125}I an isotopically enriched target of ^{125}Te would be irradiated (^{125}Te(p,n)^{125}I). However since the target also contains an often undetermined and batch-specific amount of ^{128}Te impurity, traces of (non-radioactive) ^{127}I are produced simultaneously (^{128}Te(p,2n)^{127}I). Furthermore, after irradiation the iodine is washed from the surface of the blank using a solution which inevitably contains some iodine. While the specific activity of commercial iodine products is provided as "carrier free," it is technically impossible to produce an isotopically pure preparation of the radioisotope, and "carrier free" means that no free iodine was deliberately added to the preparation in addition to these necessary and unknown sources, although a multiple molar excess of nonradioactive iodine may be present (this phenomenon is not restricted to iodine isotopes). For instance, whereas the maximum theoretical specific activity of ^{125}I is 642.7 GBq/mg iodide, the product we purchased was rated as >600 GBq/mg. The corresponding values for ^{131}I are 4587 GBq/mg (max. theor.) and 740 GBq/mg (product specification). For a technical description of the processes involved, see Bonardi et al. ***(20)***. For an estimate of the labeling stoichiometries obtained by us, see the legend to Table 3. For most researchers there is no practical way to quantify the isotopic purity of the small highly radioactive mixture that is commercially delivered. When dealing with very small amounts of protein, such as from clinical microdissection samples (***7***), we sometimes experienced smearing (not chain formation) in the basic region of 2D-PAGE patterns due to over-iodination caused by cold iodine in the "no-carrier-added" commercial stocks. Because these samples were extremely valuable, this led us to explore the strategy of sample pooling to generate a critical mass of protein in the labeling reactions, which produced more consistently reliable results over replicate samples (***7***). On the other hand, when sufficient sample is available the labeling conditions can be calibrated for particular batches of iodine and protein to achieve very much higher incorporated specific activity and thereby sensitivity than attained here. In hindsight we could have systematically iodinated the samples in

parallel with tenfold more specifically active iodine in the labeling reactions, which would probably have greatly improved the quantification of glycogen phosphorylase, whose signal was close to background levels in our Sample A gels and which consequently deconvoluted with errors only slightly better than background variation. While the indicative calculations of **Table 3** assume that each tyrosine reacts identically, in practice the electron density contributed to the tyrosine ring by adjacent amino acids may produce sequence-specific differences in reactivities of perhaps up to 100-fold or more in extreme cases (assuming a range of pka values of 2 units for protein tyrosine residues). These would nevertheless only contribute to overlabeling when two or more hyperreactive residues were present in one analyte protein molecule.

10. It is recommended to place 1 µL of a diluted labeled protein *from each reaction mixture used on that gel* onto the border of the blotting paper adjacent to the gel but within the area that will be imaged. The amount of radioactivity required in this signal source will vary depending upon the sensitivity of your imager. It should not saturate the pixel distribution of the imager during the exposure period you choose, yet is useful when >50% saturation is achieved: 60 Bq is a useful guide value. Also place 1 µL of a 1:1 mixture of both labeled proteins (i.e. 30 Bq of protein mixture labeled with each isotope in 1 µL). These optional signals provide useful controls for the deconvolution process later.
11. Unless the gels are perfectly dry they risk shattering into many pieces when the vacuum is removed, or slowly cracking after lamination. When the latter occurs between the first and second imaging scans the images are not superposable, and cannot be deconvoluted! Although these effects can affect every experiment, the consequences are especially severe when working with a large number of gels in a matrix-designed radioactive experiment. As a rule of thumb, the gels are dry when there is no temperature difference apparent between the metal surface of the gel dryer and the drying gel as judged by (disposable gloved) hand contact.
12. DO NOT BEND IPs. The phosphor crystals of the IP are damaged when the IP bends, resulting in less sensitive regions.
13. Because the gels will be exposed twice, and both images must be processed in parallel to generate the individual isotope images, pay attention that the corner of the laminated gel is pressed so that the edges are flush with the edges of the exposure cassette at one particular corner for both exposures.
14. It is highly advisable to expose the same gel with the same IP in the exact same orientation for both scans in your experiment. In case the surface of the IP has been damaged and exhibits differing sensitivities, this can be largely eliminated by imaging the same gel region with the same IP surface in both

scans. Each IP has one corner cut away to help with systematic orientation of the IP with respect to the gel.

15. Exposure in a lead- and copper-lined metal exposure cabinet (“safe”) significantly decreases background signals from environmental radiation, permitting the more accurate detection of low intensity signals from the gels. Our version was purchased from raytest GmbH, Straubenhard.
16. You should have the imaging device in a room with low light level, such as drawn blinds or a room without windows. (You will require a few candle power of light to see what you are doing though, such as perhaps the light of the monitor of your PC that runs the FLA3000.) The signal on the IPs is extremely sensitive to light. Only remove the exposed IP from the cassette with the room lights off. The FLA3000 should be able to operate in light while scanning. However we have very occasionally had problems that the sealings of the scanner were not tight and some measurements were interrupted, destroying that exposure and potentially costing a repeat of the whole experiment. So we advise to also keep the room lights off while the imager is scanning.
17. To avoid bending the IP while removing it, a suction device may be useful to lift the plate the first few millimeters **(Fig. 7).**
18. For the FLA3000 the IP has a magnetic backing and is placed into an IP stage for scanning. The IP stage is marked with a grid of letters and numbers. To assist with image orientation, we suggest that the cut corner of the IP should go towards the A9 corner as shown **(Fig. 8).** Make sure that the IP sits squarely in the stage, and that the edges are as close as possible to both sides.
19. Make sure that the IP stage is sitting exactly in the FLA3000. If it is crooked it can destroy the motor of the FLA3000 and/or the IP stage if the stage does not sit flatly as the FLA3000 is trying to move it into the scanning area. Make sure the arrow between “H” & “I” on the IP Stage lines up with the arrow on the FLA 3000 **(Fig. 9).**
20. It may be advisable to make two or more successive measurements without removing the IP stage and IP from the imager if some regions of the gel exhibit saturated pixel intensity. You will need to establish an appropriate systematics of data structure and file nomenclature.
21. We employed only a three week decay period here because of study schedule restraints for the ABRF PRG2006 study. The ability to accurately deconvolute signals containing low intensities of ^{125}I will be increased by permitting longer decay times, whereby the actual number of randomly decayed ^{125}I atoms is given time to approach the theoretically calculated expected number more precisely. Three to five weeks decay period is a practical compromise and should be empirically determined by each laboratory for itself.

22. ProteoTope experiments resemble somewhat the activity of the warrior, with shorter periods of activity interspersed by longer periods of waiting (3–5 weeks) for radioactive decay. However different temporally overlapping experiments can be sequentially coordinated such that the overall throughput is continuous and considerable, and has proved to be adequate for our industrial applications. Although iodine is not a natural component of most proteins, it can be efficiently introduced into proteins from any source by the post harvest labeling method ***(19)***, including clinical human samples. Because the differential images produced for ProteoTope rely on the different decay rates for each isotope (half lives of 59.41 days for ^{125}I and 8.04 days for ^{131}I), the same Imaging Plate can be used for both measurements without the requirement of an absorber as required by the method of Johnstone et al. ***(21)***, and thus there is no source of distortion of the spatial signal distribution or detection efficiency. The vast majority of signal from ^{125}I captured by the Imaging Plate is from the 27 keV and 31 keV photons. The Auger electron is essentially too weak to be detected. For ^{131}I the 364 keV photon does not efficiently interact with the plate crystals, so that the vast majority of signal from this isotope comes from the 810 keV beta particle. Because this beta particle interacts more efficiently with the phosphor crystals than the low energy photons from ^{125}I, the signal detected from ^{131}I is slightly more localized than the more diffuse signal for ^{125}I. However both are favorably comparable in resolution, as evident from our results in this and other cited studies. The precise composition of our imaging plates remains unpublished by the manufacturer, and there is no data available from the manufacturer or distributor on the detection efficiency of the Imaging Plates for the various types of radiation we used. Therefore the discussion of detection efficiencies in this note represents the outcome of empirical observation and deduction. We do not employ ProteoTope to obtain absolute quantitative values, but rather to obtain accurate estimates of differential protein abundances between two samples. There may be some concern that the slightly more diffuse signal pattern generated by ^{125}I may lead to problems in differential quantification between isotope channels. We neglect this since the overwhelming majority of the signal is localized close to the source for both isotopes, and because the signal intensities of the quantified areas for particular spots are normalized to each other after deconvolution of the image channels. The latter effectively corrects any systematic distortion error that might arise because of the different signal distributions for each isotope (signal volumes for the whole gel are effectively normalized across detected spots). The major source of error is statistically related to signal counting acquisition, which is dictated by the amount of radio-

activity present. Fluctuations from ideal exponential decay will result in the signal from one isotope being incorrectly deconvoluted into the channel of the other. Low intensity signals of ^{125}I are most prone to this error. Results are effectively identical whether one employs the spot detection mask for the ^{125}I or ^{131}I channel, however it is advisable to employ the one mask for both channels for calibrated quantification (as was done to generate **Fig. 4**). One could also consider employing an intensity peak-based signal redistribution algorithm on the ^{125}I signal to make it more closely resemble that of ^{131}I, however we have not found that to be necessary and prefer to work with raw distributions which generate fewer opportunities for unforeseen data-processing artifacts to arise. Therefore the simple solution of equations (1) and (2) (*see* **Note 23**) is (A) technically correct to separate the radioactive signals originating from the two respective isotopes by deconvolution, and (B) pragmatically adequate to generate the data quality reported here without subsequently correcting for the marginally different signal distributions of those isotopes.

23. The two resulting images of each gel are used to generate deconvoluted images of the signals for each isotope using simultaneous equations. Briefly, the pixel value of any pixel of the first exposure is expressed in **Eq. 1,**

$$A + B = X \tag{1}$$

and the pixel value of a given pixel of the second exposure is expressed in **Eq. 2**,

$$MA + NB = Y \tag{2}$$

where

A = the signal contribution of radiation type A (e.g., ^{125}I) measured from the first exposure,

B = the signal contribution of radiation type B (e.g., ^{131}I) measured from the first exposure,

M = decay factor for the set-specific radiation type A between the two measurements,

N = decay factor for the set-specific radiation type B between the two measurements.

X = pixel value corresponding to the radiation intensity in the first measurement,

Y = pixel value corresponding to the radiation intensity in the second measurement.

A and B are unknown and to be determined. X and Y are the measured intensities. M and N can be determined by measuring calibration samples if radioisotopic impurity is suspected, or by calculation based on theoretical decay rates and times of exposures, as performed in this study. The values for A and B can be calculated by simultaneously solving the equation system consisting of Eq. 1 and 2. This function was performed on a pixel by pixel basis using the aligned pixel matrices of both gel images by the own-developed "select_deconv_v1.1.pl" program (ProteoSys AG).

24. The use of the Imaging Plate to produce differential images is not new. Exposure to X-rays of different energies was already used to differentially visualize bone and soft tissue in clinical X-rays by some of the seminal Imaging Plate studies (***3, 4***). Johnstone et al. ***(21)*** used an absorbing sheet between source and Imaging Plate to discriminate between the energies of ^{32}P and either ^{35}S or ^{14}C tocreate differential images of proteins and nucleic acids in gels. In a related strategy,Monribot-Espagne et al. ***(22)*** made two different exposures using Imaging Plates sensitive to either ^{3}H and ^{14}C or only to ^{14}C to obtain differential 2D-PAGE images of proteins labeled with ^{3}H or ^{14}C that had been blotted onto membranes. However these methods have disadvantages relative to the ProteoTope strategy. The use of an absorbing sheet is associated with back-scatter as the beta-particles are deflected by atomic collisions within the absorber leading to altered geometry, altered detection efficiency, and spatial diffusion of the signal. Therefore the size and intensity of the signal measured for a given source with or without absorber is different, resulting in markedly reduced resolution and unaesthetic images (data not shown). The use of an absorber would have been quite advantageous, however we were unable to generate acceptable 2D images with any of them. Despite testing multiple absorbers types of varying thickness including diverse low Z materials (e.g., plastics and carbohydrates) and metals with various Z values, we have never encountered one that would produce data comparable to that shown here, and we therefore continue to employ differential radioisotopic decay rates.

    Both of the analogous imaging methods mentioned above involving differential use of either ^{32}P and ^{35}S/^{14}C, or ^{3}H and ^{14}C isotope pairs suffer from the serious drawback that the chemical labeling of different protein samples is dissimilar because the chemical distribution of the base elements in proteins differs when using ^{3}H, ^{14}C, ^{32}P or ^{35}S as labeling reagents. Therefore two images obtained from the same sample labeled by any two methods exhibits a different 2D-PAGE pattern, as discussed ***(19)***. Accordingly, slight differences between closely related samples as revealed in this study cannot be determined with those methods. By contrast, the ProteoTope strategy offers an efficient solution to the differential imaging of proteins, employing two chemically identical isotopes of radioactive iodine that produce identical 2D-PAGE patterns from the same sample

Spots		Intensity (PSL)		Statistics						Graphics
Label	Protein	A av Norm.	B av Norm.	ratio A/B	95% Confid. Intervall (Ratio)		SD (%)	SEM (%)	p-value	
BA_459	glycogen phosphorylase b	116	3143	0,038	0,024	0,051	2,2	0,6	2E-41	
BA_460	glycogen phosphorylase b	385	12999	0,030	0,019	0,041	1,9	0,5	1E-43	
BA_463	glycogen phosphorylase b	752	22321	0,034	0,021	0,047	2,2	0,6	1E-41	
BA_465	glycogen phosphorylase b	592	20828	0,029	0,021	0,037	1,4	0,3	4E-48	
BA_466	glycogen phosphorylase b	483	9710	0,051	0,033	0,068	2,9	0,7	9E-38	
BA_496	lactoperoxidase	2119	3360	0,640	0,473	0,808	11,5	2,9	8E-06	
BA_552	albumin	2211	2385	0,942	0,823	1,062	5,6	1,4	0,1471	
BA_554	albumin	4480	4695	0,970	0,828	1,112	6,7	1,7	0,5236	
BA_556	albumin	12876	13464	0,972	0,783	1,161	8,8	2,2	0,6478	
BA_557	albumin	33152	33504	1,005	0,786	1,225	9,7	2,4	0,9392	
BA_558	albumin	28955	29074	1,012	0,797	1,226	9,8	2,4	0,8662	
BA_563	albumin	10373	10659	0,989	0,792	1,186	9,2	2,3	0,8641	
BA_564	albumin	4273	4206	1,032	0,810	1,254	9,4	2,4	0,6395	
BA_565	albumin	3409	3347	1,035	0,847	1,222	8,2	2,1	0,5619	
BA_618	catalase	8311	1233	6,852	5,724	7,981	2,6	0,7	1E-36	
BA_620	catalase	46620	7599	6,236	5,294	7,178	3,0	0,7	1E-34	
BA_621	catalase	41922	8207	5,182	3,664	6,700	13,1	3,3	4E-15	
BA_622	catalase	47422	9386	5,126	3,316	6,935	13,2	3,3	5E-15	
BA_623	catalase	31824	4618	7,005	5,706	8,303	2,9	0,7	2E-35	
BA_625	catalase	13064	1979	6,708	4,846	8,569	4,1	1,0	1E-30	
BA_626	catalase	3824	655	5,936	2,638	9,234	5,0	1,3	1E-27	
BA_627	catalase	1625	338	4,887	3,660	6,114	6,3	1,6	1E-23	
BA_749	horse radish peroxidase C1	3355	2592	1,316	1,127	1,504	5,5	1,4	9E-08	
BA_764	horse radish peroxidase C1	2156	1964	1,116	0,973	1,258	5,6	1,4	0,0095	
BA_775	horse radish peroxidase C1	17034	13540	1,279	1,134	1,423	5,1	1,3	2E-07	
BA_781	horse radish peroxidase C1	2056	1331	1,569	1,331	1,807	6,3	1,6	6E-11	
BA_1068	beta casein	7312	1133	6,557	4,459	8,654	5,3	1,3	2E-27	
BA_1164	beta casein	68163	8868	7,813	6,293	9,332	2,7	0,7	1E-36	
BA_1171	beta casein	10112	1375	7,475	6,081	8,868	2,5	0,6	2E-37	
BA_1188	beta casein	2338	307	7,743	5,520	9,966	4,8	1,2	2E-29	
BA_1261	CA1 protein	3256	9058	0,365	0,326	0,405	3,9	1,0	2E-25	
BA_1271	CA1 protein	6236	16840	0,376	0,322	0,431	5,3	1,3	2E-21	
BA_1284	CA1 protein	3790	9424	0,409	0,336	0,482	6,4	1,6	6E-18	
BA_1292	CA1 protein	840	1759	0,485	0,375	0,595	8,6	2,1	2E-12	
BA_2292	ribonuclease a	1208	1059	1,160	1,034	1,286	4,9	1,2	0,0002	
BA_2321	ribonuclease a	3823	3227	1,204	1,058	1,350	5,0	1,3	1E-05	
BA_2329	ribonuclease a	11759	10411	1,148	0,989	1,307	6,2	1,5	0,0037	
BA_2369	ribonuclease a	49628	45344	1,112	0,937	1,288	7,1	1,8	0,0423	

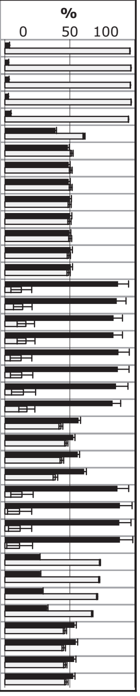
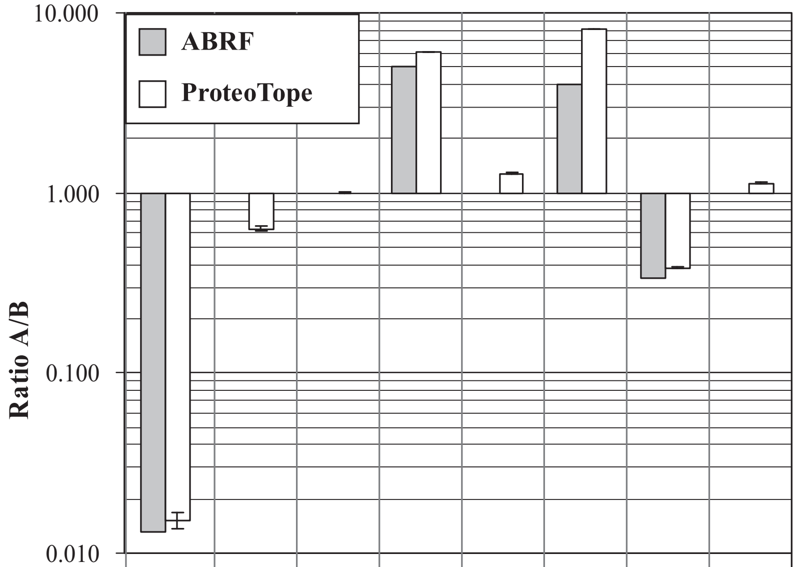


Fig. 5. Statistical results for each individual spot. The average normalized photostimulated light units (PSL) for each spot in both samples is given as ("A av Norm" and "B av Norm"). Because individual gels vary in intensity these normalized intensities were derived by applying the average of individual abundance ratios from all gels to the average sum signal intensity for each spot in columns AO-AR of spreadsheet "SelectedSpots." The corresponding ratio of A/B for relative protein abundance estimated from each spot is calculated (ratio A/B) with the accompanying 95% confidence interval for that ratio, as shown. Standard deviation (SD) and standard error of the means (SEM) are given in percentage total abundance, with the corresponding P-value for a null hypothesis of equal intensities. The bar diagrams at the right show the percentage signal for A (black bars) and B (light bars). Error bars show SEM.

in the polyacrylamide matrix of dried 2D-gels, with effectively zero post-electrophoretic sample loss.

25. Although our differential ratio estimates were the most accurate 2D-PAGE results submitted to the ABRF study by any participant, and comparable to differential isotope mass spec methods, unfortunately the abundance ratio estimates of some proteins corresponded to those given by the ABRF only within reasonably large error margins for our liking **(Fig. 6)**. However the observations discussed below lead us to conclude that the major source of



Protein	**GP**	**LP**	**BSA***	**C**	**P**	**BC**	**CAl**	**R**
% Error	**16**	**37**	**0***	**22**	**29**	**103**	**16**	**13**

Fig. 6. Comparison of ProteoTope abundance ratios with those given by the ABRF. Abundance ratios submitted for weighted mean estimates from **Fig. 5** (ProteoTope) compared to the abundance ratios published by the ABRF after the study. Error bars show Standard Errors of Means. Protein name abbreviations follow **Fig. 4.** ABRF ratios for A/B (with submitted ProteoTope ratio) were GP, 0.013 (0.015); LP, 1.000 (0.631); BSA, 1.000 (1.000*); C, 5.000 (6.106); P, 1.000 (1.287); BC, 4.000 (8.104); CA1 0.333 (0.385); R, 1.000 (1.129). The absolute percentage errors of the submitted ProteoTope estimates of these ratios relative to the ABRF values are presented underneath the protein name abbreviations (% Error). The asterisk associated with BSA values indicates perfect value of 1:1 because this protein was used to normalize the signals from each radio-isotope across all gels.

discrepancy arose at the point of sample resolubilization, rather than during the quantitative analysis itself. There were two estimates of abundance ratios between Samples A and B that warrant particular discussion in this context: Lactoperoxidase and Beta Casein. For Lactoperoxidase our estimated ratio A/B was 0.63:1 ($P = 4\times10^{-6}$), rather than the ABRF value of 1:1. (We explicitly and categorically have no reason to presume incorrect sample mixing by the ABRF.) This protein exhibited generally weak incorporation of radioactivity relative to that of a silver stained gel of the

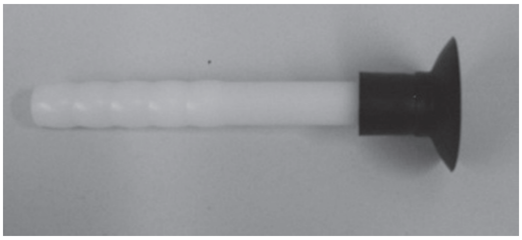


Fig. 7. A suction device to remove IPs from exposure cassettes without bending them. The handle is a hollow tube. Suction is created by hole at the blocking the upper (left in the image) end with a finger. This provides sufficient force to lift the IP high enough out of the cassette that its edges can be accessed with the fingers. The suction device was purchased from raytest GmbH.

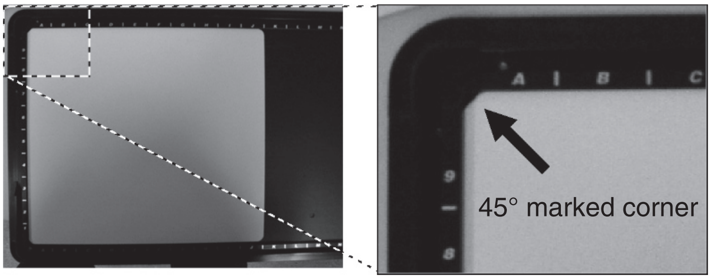


Fig. 8. Recommended orientation of the Fuji IP in the FLA3000 IP stage. The left panel shows an IP in the IP stage ready for scanning. The right panel shows an enlargement of the upper left corner, where the diagonally removed corner of the IP is oriented. Both edges of the IP should be exactly flush with the walls of the IP stage.

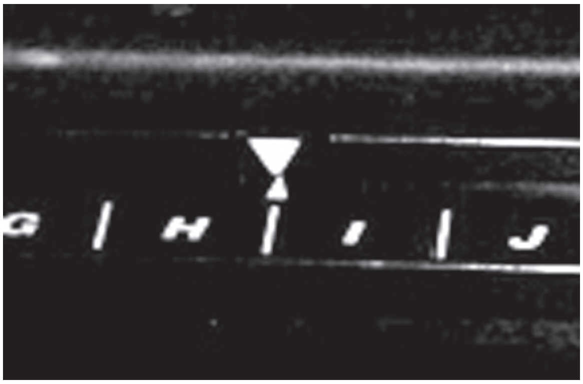


Fig. 9. Positioning the FLA3000 IP stage into the FLA3000 imager. The small arrow between H & I on the reverse of the IP stage should align with the large arrow on the FLA3000 imager.

same samples ***(10)***, which was reflected by the rather large 95% confidence limits of **Fig. 5** (0.473 – 0.808). However, in light of the result for Beta Casein, we conclude that the abundance of this protein had probably been altered in at least one sample in some manner prior to taking the sample aliquots for the radiolabeling step.

For Beta Casein our estimate of the A/B abundance ratio contained >100% error relative to the nominal ABRF value (weighted average estimate 8.104:1 compared to ABRF value 4:1). None of the 95% confidence limits for any of our four quantified spots approach a ratio of 4 **(Fig. 5)**, and a t-test against the null hypothesis of a 4:1 ratio shows a highly significant difference ($P = < 0.00001$). Because of our rigorous experimental design **(Fig. 1A**) we can therefore be statistically quite confident that the abundance of this protein was altered in at least one sample between the time the samples were mixed in the laboratory of the ABRF and the time that the two identical aliquots were taken from each resolubilised sample before iodination in our laboratory. Note that the alteration(s) did not occur during or after radio-iodination of the samples (during the ProteoTope analysis itself), which would have increased the measured variability and reduced the significance of the results. There is no other (persuasive) argument for the reproducible estimation of these observed abundance ratios being obtained after labeling the proteins with two different radioiodine isotopes used in eight labeling reactions across sixteen inverse replicate gels **(Fig. 1A).** It must be emphasized that although the estimates of abundance ratios that we submitted were associated with undesirably high deviations from the values provided by the ABRF **(Fig. 6),** we could identify the probable cause of the discrepancy because of our stringent experimental design. It is that process and its attributes, rather than the submitted abundance ratios, which provide the main technical features of the data quality generated by the ProteoTope method.

The most probable source of this sample alteration is sample handling prior to labeling in our laboratory, or possibly during shipment. One obvious source of potential variability is the resolubilization of the desiccated pellet. There was no opportunity to optimize this process because of the imposed time limit, and the resolubilization of desiccated proteins is not standardized in our laboratory. We would normally recommend transport of proteins as frozen SDS solutions or tissue sections, although this is associated with higher costs. We were the only participant to resolubilize these desiccated proteins in boiling SDS buffer, suggesting that this approach may have been suboptimal for these samples, however further experiments would be necessary to draw a conclusion. The fact that at least one of the samples was apparently suboptimally resolubilized underlines that this was a real-life one-off experiment. This further emphasizes the requirement for scrupulously standardized sample handling procedures when working with physico-chemically labile protein species. Nevertheless, the confidence with which we may draw these conclusions stems from our rigorous experimental design and the high quality quantitative data generated.

This introduces a further consideration of data quality assessment that merits brief discussion. In this study we employed the somewhat unusual number of 16 SDS-PAGE gels, generating 32 images, to compare two samples. The entire ProteoTope quantitative analysis consumed just 25% of each sample (nominally $20\,\mu g = 2{\times}2{\times}5\,\mu g$), and therefore we could have generated 64 such differential 2D-PAGE gels using the same design with the nominally 80 μg protein in each sample. This high number of accurate measurements gave rise to extraordinarily precise and highly significant differential quantifications of the various abundance ratios in the samples we measured (after they had apparently been differentially solubilized). For instance, Ribonuclease A was a protein with a nominal 1:1 mixture ratio given by the ABRF. The ratios estimated for the four individual spots of this protein in **Fig. 5** ranged from 1.112 to 1.204, generating *P*-values ($n = 16$ per spot) between 0.042 to 1×10^{-5}. Once again, because of the meticulous experimental design, we can conclude that there was a slight difference (relative to BSA levels) in the abundance of this protein between the samples before the replicate aliquots were taken for iodination. A similar situation would apply for Horse Radish Peroxidase. Alternatively, there may have been a difference in BSA levels, against which signal intensities for both radio-isotopes were normalized. Consistent with the latter possibility, normalization of spot intensities to assumed 1:1 values of Peroxidase and Ribonuclease rather than 1:1 for BSA did give somewhat better approximation of abundance ratio estimations to those provided by the ABRF. In summary, although the extraordinarily stringent level of statistical certainty obtained here is seldom required, the availability of an analytical detection method with these technical parameters can be advantageous, especially when smaller numbers of replicates are performed on limited amounts of valuable material.

26. The weakest average protein spot intensity in this study (Spot BA_459 sample A) was 116 photo stimulated luminescence units (PSL) (11, 12). The highest was 94973 PSL (BA_2369), representing a dynamic range of 800-fold measured between protein signals in this experiment. The strongest signal was on gel 2 which had an intensity of 16800-fold above measurement background and occupied almost 60000 levels of the 16 bit data spectrum. The FLA3000 we used is a well characterized instrument whose technical specifications are available from the manufacturer (e.g. http://fujifilmlifescienceusa.com/assets/pdf/fla3000brochure.pdf).

Essentially, laser induced PSL events are recorded by a photomultiplier detector. Data are then written to a floating point matrix file which stores data logarithmically over the 65536 bits. The linear dynamic range of the Imaging Plate, although intrinsically vastly superior to alternative methods, is presently limited by the imaging device (data not shown). Briefly the Imaging Plate has at least 250-fold better dynamic range than is currently exploited by the FLA3000 imaging device.

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Quantification of Proteins on Polyacrylamide Gels

Bryan John Smith

1. Introduction

Quantification of proteins is a common challenge. There are various methods described for estimation of the amount of protein present in a sample, for example, amino acid analysis and the bicinchoninic acid (BCA) method (*see* Chapters 83 and 3). Proteins may be quantified in sample solvent prior to electrophoresis and can provide an estimate of total amount of protein in a sample loaded onto a gel ***(1,2)***. These methods quantify total protein present but not of one protein in a mixture of several. For this, the mixture must be resolved. Liquid chromatography achieves this, and the various proteins may be quantified by their absorbancy at 220 nm or 280 nm. Microgram to submicrogram amounts of protein can be analyzed in this way, using microbore high-performance liquid chromatography (HPLC). A method has been published whereby theoretical extinction coefficients may be calculated for peptides, based on their sequence, though it is probably less suited to intact proteins because of the influence on UV aborption of longer sequence and structure. ***(3)***. Problems may occur if particular species chromatograph poorly (such as hydrophobic polypeptides on reverse-phase chromatography).

An alternative is polyacrylamide gel electrophoresis (as the mixture-resolving step) followed by protein staining and densitometry. Exceptions are small peptides that are not successfully resolved and stained in gels, and which are more suited to chromatography ***(3)***. Microgram to submicrogram amounts of protein (of more than a few kilodaltons in size) may be quantified in this way. Not every protein stain is best suited to quantification in gels, however. The popular noncolloidal Coomassie gel stains such as PAGE blue ‘83 are not suitable. As discussed by Neuhoff et al. ***(4)***, staining by Coomassie dye is difficult to control—it may not fully penetrate and stain dense bands of protein, it may demonstrate a metachromatic effect (whereby the protein–dye complex may

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show any of a range of colors from blue through purple to red), and it may be variably or even completely decolorized by excessive destaining procedures (because the dye does not bind covalently). As a consequence, stoichiometric binding of Coomassie dye to protein is commonly not achieved. Sensitive silver stains are commonly used to detect rare proteins at levels at levels of the order of 0.05 ng/mm^2 in two-dimensional (2-D) gels, for example, but they, too, are not suitable for quantification. For various reasons, proteins vary widely in their stainability by silver—some do not stain at all, others may show a metachromatic effect. Use of silver staining for quantification is complicated by other factors, too—such as the difficulty in obtaining staining throughout the gel, not just at the surface. This subject is reviewed at greater length in **ref.** *5*, but in summary it may be said that while silver stain methods have great sensitivity, they are problematical for quantification purposes.

One quantitative staining method generates negatively stained bands ***(6)***. The technique generates a white background of precipitated zinc salt, against which clear bands of protein (where precipitation is inhibited). This may be viewed by dark field illumination. The gel may be scanned at this stage, the negative staining being approximately quantitative (for horse myoglobin) in a range from about 100 ng/mm^2 to 2 µg/mm^2 or more. Sensitivity may be improved by staining of the background by incubation with tolidine. The method was described for 12% and 15% T gels, but the degree of zinc salt precipitation and subsequent toning is dependent upon %T and at closer to 20%T the toning procedure may completely destain the gel, restricting the usefulness of the method.

A number of other quantitative staining methods have been discussed in the literature. Notably, Neuhoff et al. ***(4)*** have made a thorough study of various factors affecting protein staining by Coomassie Brilliant Blue G-250 (0.1% [w/v] Color Index number 42655 in 2% [w/v] phosphoric acid, 6% [w/v] ammonium sulfate). This colloidal stain does not stain the background in a gel and so washing can give crystal-clear backgrounds. A method derived from that of Reisner et al. ***(7)*** has proven useful for quantification of proteins on gels. The stain used is 0.4% (w/v) Brilliant Blue G250 in 3.5% (w/v) perchloric acid. This method is described below.

O'Keefe ***(8)*** described a method whereby cysteine residues are labeled with the thiol-specific reagent monobromobimane. On transillumination (at $\lambda = 302$ nm) labeled bands fluoresce. However, the method requires quantitative reaction of cysteines, which may be totally lacking in some polypeptides, and a nominal detection limit of 10 pmol of cysteine is claimed. Better luminescent stains have been developed by Molecular Probes—the Sypro family of dyes. The Sypro Ruby stain is possibly the most sensitive of these, approaching the sensitivity of silver stains. The method for use of this stain is given below.

2. Materials

2.1. Colloidal Coomassie Brilliant Blue G Method

1. A suitable scanning densitometer, for example, the Molecular Dynamics Personal Densitometer SI with Image Quant software or the Bio-Rad Fluor S MultiImager with Multi Analyst software. Such equipment can scan transparent objects such as wet gels, gels dried between transparent films, and photographic film, and digitize the image that may then be analyzed.
2. Protein stain: 0.4% (w/v) Coomassie Brilliant Blue G (C.I. 42655; Sigma, code B0770, 90% by elemental analysis) in 3.5% (w/v) perchloric acid (*see* **Note 1**). Stable for months at room temperature, in the dark. Beware the low pH of this stain. Use personal protective equipment (coat, gloves, safety glasses). Use fresh, undiluted stain, as supplied. For best results, do not re-use the stain.
3. Destaining solution: Distilled water.

2.2. Sypro Ruby Method

1. Equipment for scanning fluorescent bands on gels, for example, the Bio-Rad Fluor S MultiImager with Multi Analyst software, which can be used to photographically record or scan wet gels, allowing repeated scanning procedures (as staining or destaining proceeds). Simple viewing of stained bands may be done under a hand-held 300-nm UV lamp or on a transilluminator. Protect eyes with UV-opaque glasses. The Sypro dye may be excited at 280 or 450 nm and emits at 610 nm.
2. Protein stain: Sypro Ruby gel stain (Molecular Probes, product number S-12000 or S-12001) (*see* **Note 2**). This stain is stable at room temperature in the dark. Exact details of the stain are not revealed but according to the manufacturer, the stain contains neither hazardous nor flammable materials. Use fresh, undiluted stain only.
3. Destain: Background may be reduced by rinsing in water.
4. Clean dishes, free of dust that might contribute to background staining.

3. Method

3.1. Colloidal Coomassie Brilliant Blue G Method

1. At the end of electrophoresis, wash the gel for a few minutes with several changes of water (*see* **Note 3**), then immerse the gel (with gentle shaking) in the colloidal Coomassie Brilliant Blue G protein stain. This time varies with the gel type (e.g., 1.0–1.5 h for a 1–1.5 mm thick sodium dodecyl sulfate [SDS] polyacrylamide gel slab), but cannot really be overdone. Discard the stain after use, for its efficacy declines with use.
2. At the end of the staining period, decolorize the background by immersion in distilled water, with agitation, and a change of water whenever it becomes colored. Background destaining is fairly rapid, giving a clear background after a few hours (*see* **Notes 4** and **5**).

3. Measure the extent of blue dye bound by each band by scanning densitometry. Compare the dye bound by a sample with those for standard proteins run and stained in parallel with the sample, on the same gel (*see* **Notes 6–8** and **11–17**).

3.2. Sypro Ruby Method

1. At the end of electrophoresis, rinse the gel in water briefly, put it into a clean dish and then cover it with Sypro Ruby gel stain solution. Gently agitate until staining is completed, which may take up to 24 h or longer (*see* **Note 9**). Overstaining will not occur during prolonged stained. Do not let the stain dry up on the gel during long staining procedures. Discard the stain after use, for it becomes less efficacious with use. During the staining procedure the gel may be removed from the stain and inspected under UV light to monitor progress. If the staining is insufficient, the gel may be replaced in the stain for further incubation.
2. Destain the background by washing the gel in a few changes of water for 15–30 min.
3. Measure the extent of dye bound, that is, the luminescence, by each band by scanning. Compare the dye bound by a sample with that for standard proteins run and stained in parallel with the sample, on the same gel (*see* **Note 10**).

4. Notes

1. The Coomassie Brilliant Blue G stain mixture may be readily made from the components but a commercially-available alternative is the GelCode blue stain reagent from Pierce (product no. 24590 or 24592). Details of the stain components are not divulged, other than they also include Coomassie (G250), but the stain is used in the same way as described for the reagent prepared as described above. Pierce recommend that their GelCode stain is agitated before use, to disperse any dye aggregates that may have formed therein.
2. Molecular Probes do not reveal the components of their reagent. Although more sensitive than the blue stain, it does require UV irradiation for detection.
3. The water wash that precedes staining by Coomassie Brilliant Blue G is intended to wash away at least some SDS from the gel, and so speed up destaining of the background. However, it should be remembered that proteins are not fixed in the gel until in the acidic stain mixture and consequently some loss of small polypeptides may occur in the wash step. Delete the wash step if this is of concern, or employ a fixing step immediately after electrophoresis, for example, methanol/glacial acetic acid/water: 50:7:43, by volume for 15–30 min, followed by water washing to remove the solvent and acid.
4. Destaining of the background may be speeded up by frequent changes of the water, and further by inclusion in this wash of an agent that will absorb free dye. Various such agents are commercially available (e.g.,

Cozap, from Amika Corp.), but a cheap alternative is a plastic sponge of the sort used to plug flasks used for microbial culture. The agent absorbs the stain and is subsequently discarded. The background can be made clear by these means, and the stained bands remain stained while stored in water for weeks. They may be restained if necessary.

5. The Coomassie Brilliant Blue G-stained gel may be dried between transparent sheets of dialysis membrane or cellophane (available commercially, e.g., from Novex) for storage and later scanning. Beware that bubbles or marks in the membrane may add to the background noise upon scanning.
6. Heavily loaded samples show up during staining with Coomassie Brilliant Blue G, but during destaining the blue staining of the proteins becomes accentuated. Bands of just a few tens of ng are visible on a 1 mm-thick gel (i.e., the lower limit of detection is $< 10\,\text{ng/mm}^2$). Variability may be experienced from gel to gel, however. For example, duplicate loadings of samples on separate gels, electrophoresed and stained in parallel, have differed in staining achieved by a factor of 1.5, for reasons unknown. Furthermore, different proteins bind the dye to different extents: horse myoglobin may be stained twice as heavily as is bovine serum albumin, although this, too, is somewhat variable. While this Coomassie Brilliant Blue G is a good general protein stain, It is advisable to treat sample proteins on a case by case basis.
7. It is a requirement of this method that dye is bound stoichiometrically to polypeptide over a useful wide range of sample size. The staining by Coomassie Brilliant Blue G may be quantitative, or nearly so, from about 10 to $20\,\text{ng/mm}^2$ up to large loadings of $1\text{–}5\,\mu\text{g/mm}^2$. Concentrated protein solutions may be diluted to fall within the useful range. A general point may be made, that whatever method is used for quantification, it should be checked with the protein of interest, to confirm that staining is linear (quantitative) over the range of sample size being used. The stoichiometry of dye binding is subject to some variation, such that standard curves may be either linear or slightly curved, but even the latter case is acceptable provided standards are run on the same gel as samples.
8. Because performance of the Coomassie Brilliant Blue G stain is variable it essential to run and stain standards and samples on the same gel, and to do so in duplicate.
9. The Sypro Ruby method allows for periodic observation and further staining if required. It can be the case that for small loadings, however, prolonged staining (24 h or more) may be required to obtain best sensitivity. The stained gel may be dried for storage but can then lose luminescence.
10. The Sypro Ruby method is subject to the same sort of variation as the Coomassie Brilliant Blue G method, from gel to gel with standard curves being linear or slightly curved. Generally the Sypro Ruby method is more

sensitive than the Coomassie Brilliant Blue G method, for instance about fivefold more so for bovine serum albumin. However, protein to protein variation may apparently be greater in the case of the Sypro Ruby stain. For instance, horse myoglobin binds about 10-fold less dye than bovine serum albumin does. Thus in one experiment, the minimum amount of bovine serum albumin detectable after Sypro Ruby staining was about 5 ng/mm^2 (about four- to fivefold more sensitive than samples stained in parallel by the Coomassie Brilliant Blue G method), whereas the minimum amount of horse myoglobin detectable was about 50 ng/mm^2 (similar to that detectable by the Coomassie Brilliant Blue G stain). It is therefore recommended that samples and standards are run and stained in parallel on the same gel, in duplicate.

11. Best quantification is achieved after having achieved good electrophoresis. Adapt the electrophoresis as necessary to achieve good resolution, lack of any band smearing or tailing, and lack of retention of sample at the top of the gel by aggregation. For stains where penetration of dense bands may be a problem, avoid dense bands by reducing the size of the loaded sample and/or electrophoresing the band further down the length of the gel (to disperse the band further) and/or use lower %T gel. Thin gels, of 1 mm thickness or less, allow easier penetration of dye throughout their thickness. Gradient gels have a gradient of pore size that may cause variation of band density and background staining. Use nongradient gels if this is a problem.
12. For absolute quantification of a band on a gel, accurate pipetting is required, as is a set of standard protein solutions that cover the concentration range expected of the experimental sample. Ideally, adjust the concentrations of the various standards and a sample so that the volume of each that is loaded onto the gel is the same and the need for pipet adjustment is minimized. Run and stain these standard solutions at the same time and if possible on the same gel, as the experimental sample in order to reduce possible variations in band resolution or staining, background destaining and so on. Ideally the standard protein should be the same as that to be quantified but if, as is commonly the case, this is impossible then another protein may be used (while recognizing that this protein may bind a different amount of dye from that by the experimental protein, so that the final estimate obtained may be in error). The standard protein should have similar electrophoretic mobility to the proteins of interest, so that any effect such as dye penetration, due to pore size, is similar. Make the standard protein solution by dissolving a relatively large and accurately weighed amount of dry protein in water or buffer, and dilute this solution as required. If possible, check the concentration of this standard solution by alternative means (say amino acid analysis). Standardize treatment of samples in preparation for electrophoresis—treating of sample

solutions prior to SDS-PAGE may cause sample concentration by evaporation of water for instance. To minimize this problem heat in small (0.5 mL) capped Eppendorf tubes, cool, and briefly centrifuge to take condensed water back down to the sample in the bottom of the tube.

13. When analyzing the results of scanning, construct a curve of absorbency vs protein concentration from standard samples and compare the experimental sample(s) with this. Construct a standard curve for each experiment.
14. If comparing the abundance of one protein species with others in the same sample, then standards are not required, provided that no species is so abundant that dye binding becomes saturated. Be aware that such relative estimates are approximate, as different proteins bind dye to different extents.
15. It is sometimes observed in electrophoresis that band shape and width are irregular—heavily loading a gel can generate a broad band that may interfere with the running of neighboring bands, for instance. It is necessary to include all of such a band for most accurate results.
16. Avoid damage to the gel—a crack can show artificially as an absorbing band (or peak) on the scan. Gels of low %T are difficult to handle without damage, but they may be made tougher (and smaller) by equilibration in aqueous ethanol, say, 40% (v/v) ethanol in water, 1 h or so. Too much ethanol may cause the gel to become opaque, but if this occurs merely rehydrate the gel in a lower % (v/v) ethanol solution. A gradient gel may assume a slightly trapezoid shape upon shrinkage in ethanol solutions—this makes scanning tracks down the length of the gel more difficult. When scanning, eliminate dust, trapped air bubbles, and liquid droplets, all of which contribute to noisy baseline.
17. Methods have been described for quantification of submicrogram amounts of proteins that have been transferred from gels to polyvinylidene difluoride or similar membrane (e.g., ***9***). A Sypro dye blot stain equivalent to the method described above for gels is available from Molecular Probes. Note, however, that transfer from gel to membrane need not (indeed, usually does not) proceed with 100% yield, so that results do not necessarily accurately reflect the content of the original sample.

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Using SDS-PAGE and Scanning Laser Densitometry to Measure Yield and Degradation of Proteins

Aaron P. Miles and Allan Saul

1. Introduction

A variety of techniques are available for the quantification of proteins, their degradation products and other impurities. Densitometry is particularly useful due to its sensitivity, accuracy and versatility, and it can be applied to proteins in gels or on membranes and used with numerous detection methods. In addition to being accurate, sensitive and reproducible, the technique is cost-effective, simple, and does not require a high degree of specialized training, yet provides technical advantages over other available tools. For example, Vuletich and Osawa showed that densitometry following SDS-PAGE and electroblotting was 20-fold more sensitive than HPLC at detecting oxidatively modified myoglobin ***(1)***. In addition, Morçöl and Subramanian reported the development of a sensitive Ponseau-S-stained dot blot/densitometric protein assay that was less subject to interference by pH, detergent and other reagents than the commonly used Bradford protein assay ***(2)***. More recently, Zhu et al. and Miles et al. reported a quantitative densitometric method that measures host cell derived impurity levels on immunoblots of recombinant proteins expressed in *E. coli* ***(3, 4)***. This method was shown to be at least 20 times more sensitive than a commercially available ELISA kit designed to measure host cell impurity levels ***(3)***. Other groups have reported on the utility of gel or fluorograph densitometry, using multiple detection methods, in carbohydrate analysis ***(5)***, taxonomic and forensic applications ***(6)***, as a clinical diagnostic tool in Balkan nephropathy ***(7)*** and in the analysis of high density lipoproteins ***(8)***, as a means of quantifying relative levels of excretory-secretory polypeptides synthesized *in vitro* by *Schistosoma mansoni* daughter sporocysts ***(9)***, and for analyzing adsorption of proteins and an oligodeoxynucleotide in Alhydrogel®-based malaria vaccine candidates ***(10)***.

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As a typical example we describe here the analysis of recombinant proteins by SDS-PAGE and scanning laser densitometry. This approach offers advantages over other techniques for two main reasons: 1) proteolytic degradation is common among these proteins, and this method is able to accurately quantify it in both minute and large amounts, and 2) the common constituents of fermentation and refolding tanks and purification processes do not cause interference, so it can be used at any stage throughout production and purification. Analysis of a recombinant malarial protein at two different stages of purity will be used to illustrate this method.

2. Materials

1. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) equipment.
2. Coomassie Blue staining solution: 40% methanol, 10% glacial acetic acid, two PhastGel Blue R tablets (Amersham Biosciences, Piscataway, NJ) per liter.
3. Coomassie Blue destaining solution: 5% methanol, 10% glacial acetic acid.
4. 0.45-micron filtration unit.
5. Concentrated, diafiltered test protein (Pvs25H) fermentation culture supernatant.
6. Purified test protein (Pvs25H) solution.
7. Personal Densitometer SI with ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA, ***[11]***).

3. Methods

The methods described below outline (1) the running of SDS-PAGE gels followed by visualization with Coomassie Blue, and (2) scanning and analyzing the gels using laser densitometry and ImageQuant software.

3.1. SDS-PAGE

1. Load the gel with samples as desired, and run according to the manufacturer's instructions.
2. Filter the staining solution using a 0.45 μm filtration unit (*see* **Note 1**).
3. Add staining solution to the gel, bring to a boil in a microwave oven, and agitate for 30 min (*see* **Notes 2** and **3**).
4. Pour off staining solution, add destaining solution, bring to a boil, and agitate for 15 min (*see* **Note 4**).
5. Change destaining solution, bring to a boil again, and continue agitation for 15 more min.
6. Change destaining solution once more, bring to a boil, and continue destaining until gel background is clear or almost clear, approximately 10 min. Take care not to overly destain the gel as minor protein bands can disappear (*see* **Note 5**).
7. Place the gel in water until scanned. **Fig. 1** shows a nonreduced 4–20% Tris-glycine SDS-PAGE gel that contains standards of purified Pvs25H (a recombinant form of

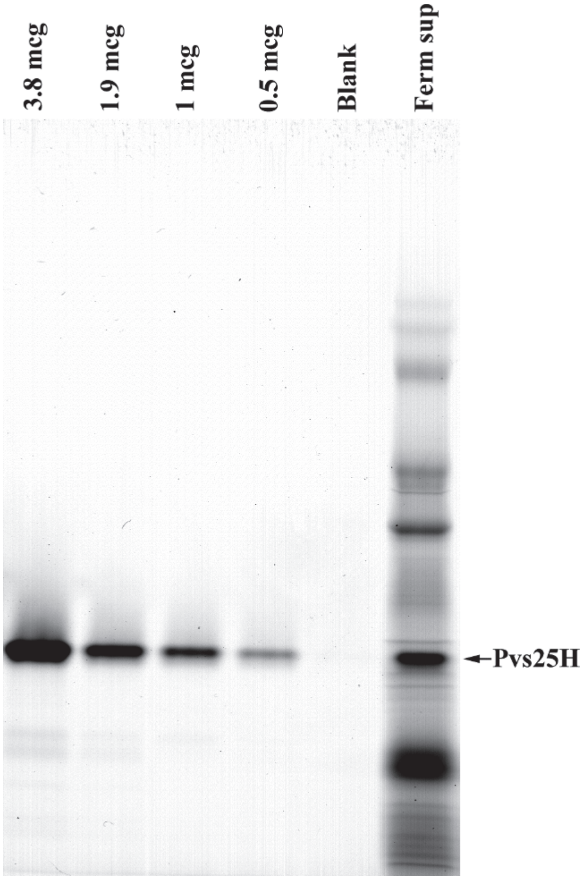

Fig. 1. SDS-PAGE of Pvs25H Fermentation Culture Supernatant with Purified Pvs25H Standards. Nonreduced 4–20% Tris-glycine SDS-PAGE Coomassie Blue-stained gel showing purified Pvs25H standards and concentrated, diafiltered fermentation culture supernatant. Purified Pvs25H was loaded in the indicated amounts for comparison to Pvs25H in the fermentation supernatant.

the 25 kDa ookinete suface protein from the malaria parasite *Plasmodium vivax*, ***[12]***) and a concentrated, diafiltered fermentation culture supernatant of the same protein. **Fig. 2** shows a reduced 15% acrylamide gel loaded with the same purified protein that was subjected to 37°C storage for up to 14 days to monitor stability (*see* **Note 6**). This protein is heavily disulfide bonded and must be reduced in order to visualize the degradation occurring over time. These gels will serve as examples of two applications of scanning laser densitometry: (1) monitoring low levels of degradation in a highly purified protein, and (2) quantifying yields of a protein recovered in a fermentation broth not yet subject to purification.

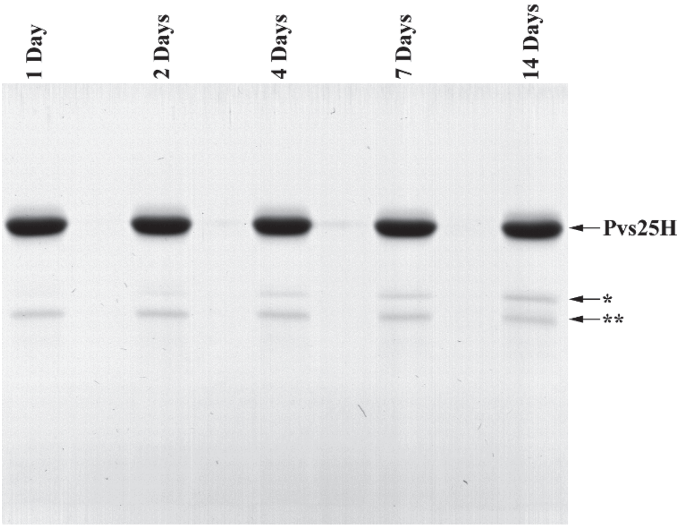

Fig. 2. SDS-PAGE of Purified Pvs25H Stored at 37°C. Reduced 15% SDS-PAGE Coomassie Blue-stained gel showing samples taken at the indicated time points during storage at 37°C. All protein loads are 2 µg, separated by blank lanes. *A minor band accumulating with time whose sequence corresponds to cleavage at a single site within Pvs25H. **A minor band (Pvs25H-derived) present in final purified Pvs25H, not increasing upon storage. This type of gel was chosen to better resolve Pvs25H, which migrates diffusely in a gradient gel (see **Fig. 1**).

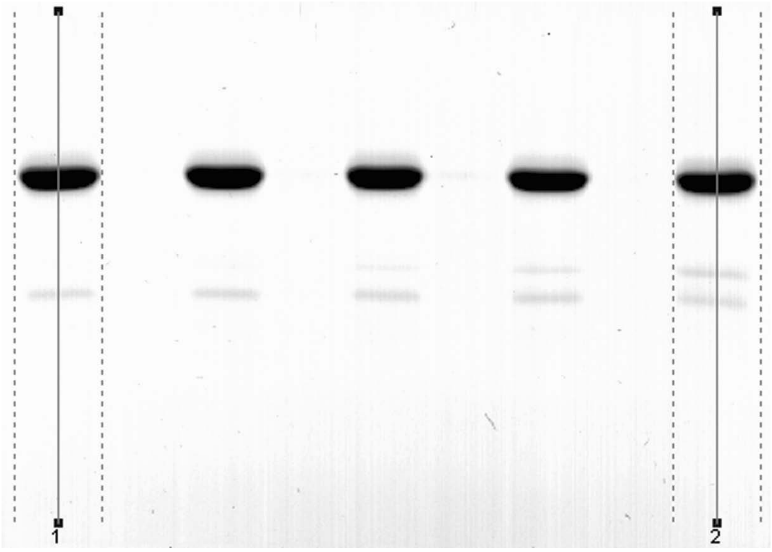

Fig. 3. Gel from Fig. 2 with Lines Drawn in ImageQuant for Analysis. Shown is the same gel as in **Fig. 2**, with lines drawn down two lanes using ImageQuant. These lines define the length and width of the areas being analyzed. Lengths and widths are adjusted to encompass all protein in each lane. These lines give rise to the line graphs shown in **Fig. 5**.

3.2. Laser Densitometry

1. Scan the gel according to manufacturer's instructions, ensuring that the lanes of the gel run straight from the top to the bottom of the scanning bed.
2. Draw an encompassing line for analysis down each lane of interest and widen the line appropriately. Include in the analyzed area of each lane enough length from top to bottom and enough width to envelope all visible protein bands. **Figs. 3** and **4** illustrate this, using the same gels as shown in **Figs. 2** and **1**, respectively.
3. Create a line graph for each line drawn. Examples are shown in **Fig. 5**, which shows representations (in optical density units) of purified Pvs25H stored for one (A) and 14 (B) days at 37°C, and **Fig. 6**, which shows Pvs25H in concentrated, diafiltered

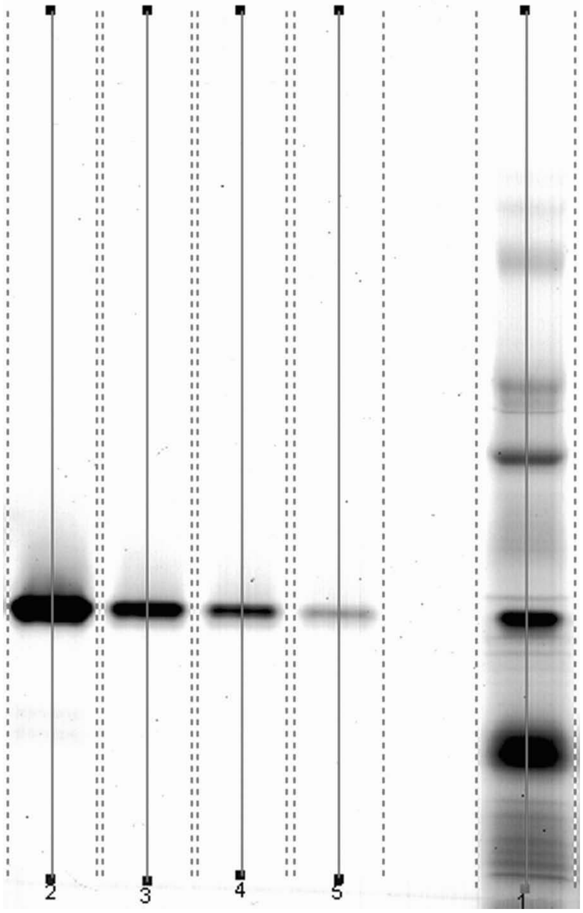

Fig. 4. Gel from Fig. 1 with Lines Drawn in ImageQuant for Analysis. Shown is the same gel as in **Fig. 1**, with lines drawn down each lane containing protein. The line drawn down the fermentation supernatant lane gives rise to the line graph shown in **Fig. 6**.

fermentation culture supernatant. Note in **Fig. 5B** the appearance of a minor degradation product over time (peak 2), and it's corresponding band in **Fig. 2**.

4. Adjust all relevant settings to give the most accurate representation of the baseline signal. Options for this within the ImageQuant software may include "automatic"

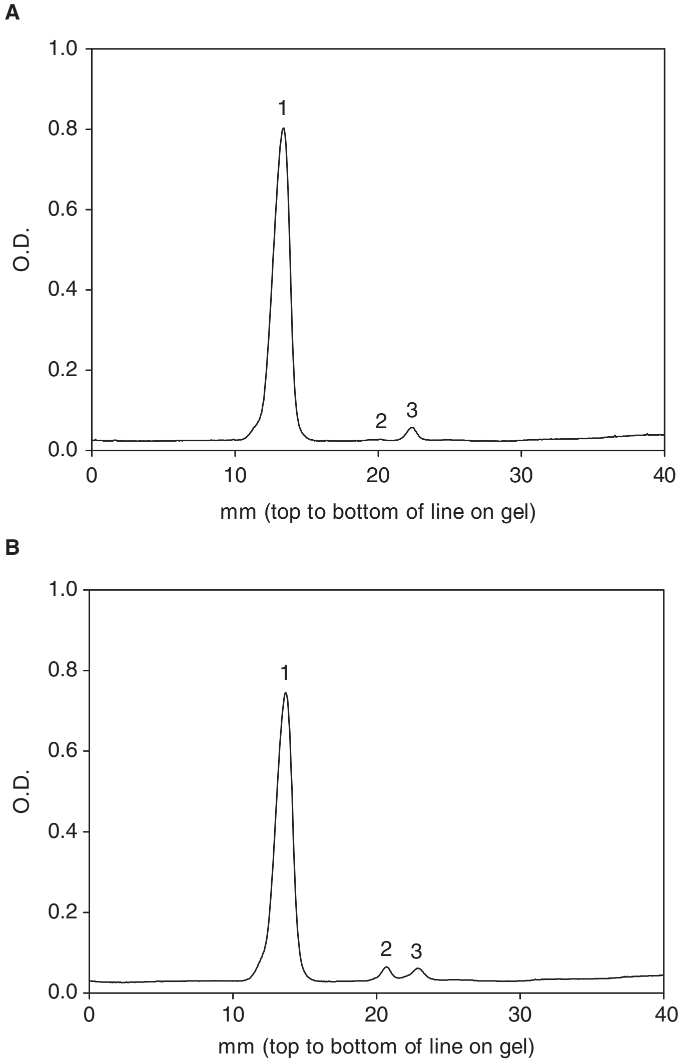

Fig. 5. Line Graphs of Pvs25H at 37°C, Days 1 and 14. The line graphs for the lines drawn in **Fig. 3** are shown, with (A) showing day 1 and (B) showing day 14. These are graphical representations of the optical density vs. millimeters measured by the scanner at each point from the top to the bottom of the drawn lines. In these cases, the baselines were detected by simply making minor adjustments to the peak detection parameters (baselines not shown). Note the increase in the area of peak 2, a minor degradation product, from day 1 to day 14.

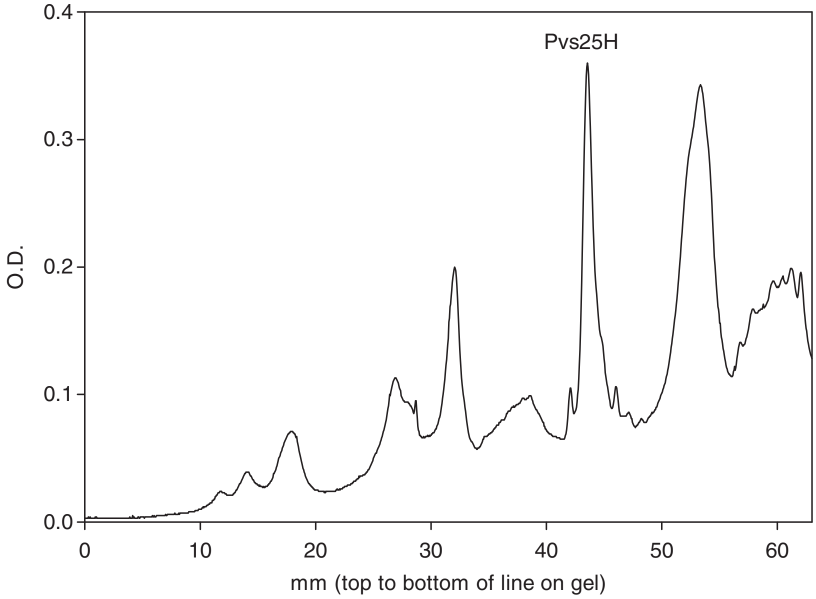

Fig. 6. Line Graph of Fermentation Culture Supernatant. The line graph for the line drawn in **Fig. 4** is shown. In this case, the baseline (not shown) was taken as the lowest point (point of lowest optical density [*see* **Note 9**]). The peak corresponding to Pvs25H is indicated. The line graphs for the standard lanes are not shown to avoid redundancy.

(the baseline is detected by the software based on manually adjusted settings to appropriately identify peaks), "lowest point" (the most transparent part of the lane is taken as the baseline), and point-by-point manipulation. There are other software programs available that allow more customized identification and tuning of the baseline and peaks (e.g., PeakFit). It is then usually necessary to redefine the peaks by hand, rather than letting the software do it, in order to fine tune where each peak begins and ends (*see* **Note 7**). Once the peaks are defined, they are integrated (i.e., the area under each peak is calculated by the software). In this way one quantifies the proportion of one or several peaks in relation to all others, and determines both the total protein in the lane and the percent of each protein of interest.

6. Using the areas generated from the integration of each Pvs25H standard peak, create a graph of area counts vs. known protein load using the appropriate software (e.g., Excel, SigmaPlot). Determine the linear range, fit a line for the data points of that range, and obtain an equation for the line. Using this method, the amount of Pvs25H in the fermentation supernatant lane of **Fig. 1** was found to be 1.49 µg. This corresponds to a total production of 500 mg from a 60 L fermentation (*see* **Note 8**). Likewise, the amount of full-length (nondegraded) purified Pvs25H was found to be decreasing with time, while a minor degradation product was increasing.

The purity of the main band dropped from 95.3% at day 1 to 94.5% by day 14. The degradation rate is thus found to be 0.06% per day at 37°C.

4. Notes

1. It is especially important when using this method to filter the Coomassie Blue stain prior to use. This eliminates large particles that otherwise stick to the gel and give rise to high background readings that interfere with quantification. Filtered stain leads to much cleaner, more transparent gels. To accelerate the filtering process and to help prevent clogging of the filter, use a conveniently sized filtration unit (e.g., 1 L) and pour the solution in slowly, allowing it to filter through, before pouring more. If too much solution is poured in all at once, large particles quickly block the filter. It is not necessary to use a 0.22 µm filter, which will become clogged more quickly anyway.
2. Microwaving allows staining and destaining to proceed much more quickly than at room temperature, with no loss in sensitivity.
3. Standard staining and destaining times must be adhered to, especially when gel-to-gel comparisons are to be made. Staining for less than 30 minutes can result in non-uniformity in the binding of the Coomassie Blue dye to the proteins, especially in bands containing large amounts of protein. This can in turn cause some underestimation in the analysis of those bands.
4. A sponge or paper towel can be added to soak up stain from the gel, but this is not necessary, especially when microwaving, as the gel will quickly destain.
5. It is unnecessary to destain the gel until the background is completely clear. A small amount of background stain will generally not be detected as a protein peak or peaks by the software, and will become part of the baseline. Higher concentrations of acrylamide in gradient gels require slightly more time to destain, but with more extended destaining times protein bands (especially low molecular weight bands) can disappear. This will affect quantification of yield and purity and should be avoided.
6. After purification of Pvs25H, it was determined that this protein is better resolved on a 15% SDS-PAGE gel than on a 4–20% gradient gel (compare **Fig. 1**, 3.8 µg lane, to **Fig. 2**, 1 Day lane). This serves to illustrate an important point to consider when performing densitometry: protein quantification is more difficult to perform when the protein runs as a diffuse band. Consequently, the error in this technique increases with poorer resolution. While a good estimate can still be achieved, peak identification can be problematic. If necessary, different gel types should be tested to find the one that best resolves each protein of interest.
7. One is often interested in the proportion of one peak (the protein of interest) in relation to all others. Defining the peaks by hand allows the peak of

interest to be isolated while, for instance, treating all other peaks as one. A percent yield is then easily calculated.

8. Scanning laser densitometry was found to be especially useful during the production of Pvs25H. This is because Pvs25H is produced as two populations of molecules that migrate well separated by SDS-PAGE (see large diffuse band beneath Pvs25H in **Fig. 1**). They do, however, co-purify during the Ni-NTA capture step. Traditional protein assays (e.g., BCA, Lowry, A_{280}) performed at this point would overestimate the yield of the target protein (labeled Pvs25H in **Fig. 1**), since these other methods are incapable of distinguishing between the two forms of Pvs25H that are produced. Densitometry makes this distinction, with the added benefit that no capture step is required before an estimate of yield is given.
9. When analyzing a sample that is not yet purified, determining the baseline is usually more difficult than when working with purified material. Each sample must be analyzed on a case-by-case basis as appropriate.

Acknowledgments

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Rapid and Sensitive Staining of Unfixed Proteins in Polyacrylamide Gels with Nile Red

Joan-Ramon Daban, Salvador Bartolomé, Antonio Bermúdez, and F. Javier Alba

1. Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most powerful methods for protein analysis ***(1,2)***. However, the typical procedures for the detection of protein bands after SDS-PAGE, using the visible dye coomassie blue and silver staining, have several time-consuming steps and require the fixation of proteins in the gel. This chapter describes a rapid and very simple method for protein staining in SDS gels developed in our laboratory ***(3–5)***. The method is based on the fluorescent properties of the hydrophobic dye Nile red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one; *see* **Fig. 1**), and allows the detection of < 10 ng of unfixed protein per band about 5 min after the electrophoretic separation. Furthermore, it has been shown elsewhere ***(6,7)*** that, in contrast to the current staining methods, Nile red staining does not preclude the direct electroblotting of protein bands and does not interfere with further staining, immunodetection and sequencing (*see* Chapter 71).

Nile red was considered a fluorescent lipid probe, because this dye shows a high fluorescence and intense blue shifts in presence of neutral lipids and lipoproteins ***(8)***. We have shown that Nile red can also interact with SDS micelles and proteins complexed with SDS ***(3)***. Nile red is nearly insoluble in water, but is soluble and shows a high increase in the fluorescence intensity in nonpolar solvents and in presence of pure SDS micelles and SDS–protein complexes. In the absence of SDS, Nile red can interact with some proteins in solution but the observed fluorescence is extremely dependent on the hydrophobic characteristics of the proteins investigated ***(8,9)***. In contrast, Nile red has similar fluorescence properties in solutions containing different kinds of proteins associated with SDS, suggesting that this detergent induces the formation of structures having

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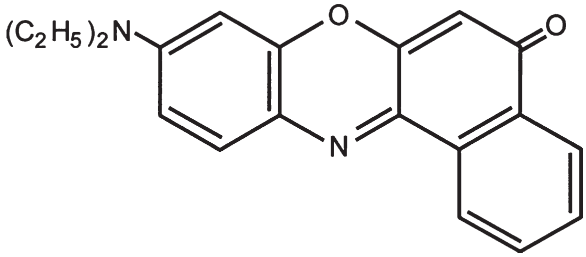

Fig. 1. Structure of the noncovalent hydrophobic dye Nile red.

equivalent hydrophobic properties independent of the different initial structures of native proteins *(3)*. In agreement with this, X-ray scattering and cryoelectron microscopy results have shown that proteins having different properties adopt a uniform necklace-like structure when complexed with SDS *(10)*. In these structures the polypeptide chain is mostly situated at the interface between the hydrocarbon core and the sulfate groups of the SDS micelles dispersed along the unfolded protein molecule.

The enhancement of Nile red fluorescence observed with different SDS–protein complexes occurs at SDS concentration lower than the critical micelle concentration of this detergent in the typical Tris–glycine buffer used in SDS-PAGE *(3)*. Thus, for Nile red staining of SDS-polyacrylamide gels *(4)*, electrophoresis is performed in the presence of 0.05% SDS instead of the typical SDS concentration (0.1%) used in current SDS-PAGE protocols. This concentration of SDS is high enough to maintain the stability of the SDS–protein complexes in the bands, but is lower than SDS critical micelle concentration and consequently precludes the formation of pure detergent micelles in the gel *(4,5)*. The staining of these modified gels with Nile red produces very high fluorescence intensity in the SDS–protein bands and low background fluorescence (*see* **Fig. 2**). Furthermore, under these conditions (*see* details in **Subheading 3.**), most of the proteins separated in SDS gels show similar values of the fluorescence intensity per unit mass. A review about the physicochemical basis of Nile red staining has been published elsewhere *(11)*.

2. Materials

All solutions should be prepared using electrophoresis-grade reagents and deionized water and stored at room temperature (exceptions are indicated). Wear gloves to handle all reagents and solutions and do not pipette by mouth. Collect and dispose all waste according to good laboratory practice and waste disposal regulations.

1. Nile red. Concentrated stock (0.4 mg/mL) in dimethyl sulfoxide (DMSO). This solution is stable for at least 3 months when stored at room temperature in a glass bottle wrapped in aluminum foil to prevent damage by light. **Handle this solution**

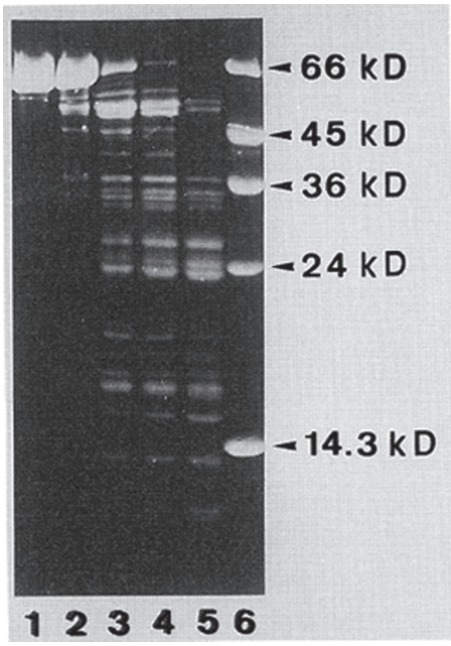

Fig. 2. Example of Nile red staining of different proteins and peptides in 0.05% SDS–15% polyacrylamide gels. The protein molecular weight markers (*lane 6*, from top to bottom: BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, trypsinogen, and lysozyme), and BSA digested with increasing amounts of trypsin (*lanes 1–5*), were stained for 5 min with a solution of Nile red in water prepared by quick dilution of a stock solution of this dye in DMSO (*see* **Subheading 3.**).

with care, DMSO is flammable, and, in addition, this solvent may facilitate the passage of water-insoluble and potentially hazardous chemicals such Nile red through the skin. Nile red can be obtained from Sigma Chemical (St. Louis, MO).

2. The acrylamide stock solution and the resolving and stacking gel buffers are prepared as described in Chapter 21.
3. 2× Sample buffer: 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.125 *M* Tris-HCl, pH 6.8; bromophenol blue (0.05% [w/v]) can be added as tracking dye.
4. 10× Electrophoresis buffer: 0. 5% (w/v) SDS, 0.25 *M* Tris, 1.92 *M* glycine, pH 8.3 (do not adjust the pH of this solution).
5. Plastic boxes for gel staining. Use opaque polypropylene containers (e.g., 21 × 20 × 7.5 and 12 × 7.5 × 7 cm for large [20 × 16 × 0.15 cm] and small [8 × 6 × 0.075 cm] gels, respectively) with a close-fitting lid to allow intense agitation without spilling the staining solution.
6. Orbital shaker for the agitation of the plastic boxes during gel staining.
7. Transilluminator equipped with midrange ultraviolet (UV) bulbs (~300 nm) to excite Nile red ***(3,4)***. A transilluminator with a cooling fan (e.g., Foto UV 300 [Fotodyne Inc., Harland, WI] or similar) is very convenient to prevent thermal

damage of the gel when long exposures are necessary (*see* **Subheading 3., step 11**). UV light is dangerous to skin and particularly to eyes. UV-blocking goggles and a full face shield, and protective gloves and clothing, should be worn when the stained gel is examined using the transilluminator. For the photography, we place the transilluminator with the gel and the photographic camera inside a home-made cabinet with opaque (UV-blocking) curtains to prevent operator exposure to UV light. Alternatively, the gel can be transilluminated inside a compact darkroom of a documentation system provided with a charge-coupled device (CCD) camera (e.g., Gel Doc 1000 from Bio-Rad Laboratories [Hercules, CA]). To avoid the problems associated with UV light, we have constructed a transilluminator that works in the visible region (~540 nm; green-light) and can also be used for the excitation of Nile red stained bands ***(12)***.

8. Photographic camera (e.g., Polaroid [Cambridge, MA] MP-4 camera) or a CCD camera.
9. Optical filters. With the Polaroid camera and UV transilluminator use the Wratten (Kodak [Rochester, NY]) filters number 9 (yellow) and 16 (orange) to eliminate the UV and visible light from the transilluminator. Place the two filters together in the filter holder of the camera so that filter 16 is on top of filter 9 (i.e., filter 16 should be facing towards the camera lens). Store the filters in the dark and protect them from heat, intense light sources and humidity. With the CCD camera and UV transilluminator we have used the filter 590DF100 (Bio-Rad). Wratten filter number 26 (red) is required for both Polaroid and CCD cameras when the bands are visualized with the green-light transilluminator.
10. Photographic films. The following Polaroid instant films can be used for the photography of the red bands (maximum emission at ~640 nm ***[3]***) seen after staining: 667 (ISO 3000, panchromatic, black-and-white positive film), 665 (ISO 80, panchromatic, black-and-white positive/negative film), and 669 (ISO 80, color, positive film). Store the films at 4°C.
11. Negative-clearing solution: 18% (w/v) sodium sulfite.
12. Wetting agent for Polaroid 665 negatives (Kodak Photo-Flo diluted at 1:600).
13. Different densitometers available commercially can be used for the densitometry of negative films. Alternatively, the images obtained with a CCD camera can be directly analyzed with the appropriate software (e.g., Molecular Analyst [Bio-Rad]).

3. Method

The method described in this part gives all the details for the staining SDS-polyacrylamide gels with Nile red. *See* **Note 1** for Nile red staining of gels without SDS. Unless otherwise indicated, all operations are performed at room temperature.

1. Typically we prepare 15% acrylamide–0.4% *bis*-acrylamide separating gel containing 0.05% SDS (*see* **Note 2**), 0.375 *M* Tris-HCl, pH 8.8. The stacking gel contains 6% acrylamide–0.16% *bis*-acrylamide, 0.05% SDS (*see* **Note 2**), 0.125 *M* Tris-HCl, pH 6.8
2. Dissolve the proteins in water, add one volume of the 2× sample buffer (**Subheading 2.3.,** *see* **Note 3**), and incubate the resulting samples in a boiling-water bath for

3 min. The samples are kept at room temperature before loading them into the gel (*see* **Note 4**).

3. Place the gel sandwiched between the glass plates in the electrophoresis apparatus, fill the electrode reservoirs with 1 × electrophoresis buffer (*see* **Note 2**), and rinse the wells of the gel with this buffer.
4. Load the protein samples (about 20 and 10 µL in large and small gels, respectively), carry out electrophoresis, and at the end of the run remove the gel sandwich from the electrophoresis apparatus and place it on a flat surface.
5. Wearing gloves, remove the upper glass plate (use a spatula) and excise the stacking gel and the bottom part of the separating gel (*see* **Note 5**).
6. Add quickly 2.5 mL of the concentrated Nile red (0.4 mg/mL) staining solution in DMSO to 500 mL of deionized water previously placed in a plastic box (*see* **Note 6**). These volumes are required for staining large gels; for small gels, use 0.25 mL of the concentrated Nile red solution in DMSO and 50 mL of water.
7. Immediately after the addition of concentrated solution of Nile red to water, agitate the resulting solution vigorously for 3 s (*see* **Note 6**).
8. Immerse the gel very quickly in the staining solution, put the lid on, and agitate vigorously (*see* **Note 6**) using an orbital shaker (at about 150 rpm) for 5 min (*see* **Note 7**).
9. Discard the staining solution and rinse the gel with deionized water (4×; about 10 s) to remove completely the excess Nile red precipitated during the staining of the gel (*see* **Note 8**).
10. Wearing gloves, remove the gel from the plastic box and place it on the UV or green-light transilluminator. Turn off the room lights, turn on the transilluminator and examine the protein bands, which fluoresces light red (*see* **Note 9**). Turn off the transilluminator immediately after the visualization of the bands (*see* **Note 10**).
11. Focus the camera (Polaroid or CCD) with the help of lateral illumination with a white lamp, place the optical filters described in **Subheading 2., item 9**, in front of the camera lens and, in the dark, turn on the transilluminator and photograph the gel (*see* **Note 10**). Finally, turn off the transilluminator.
12. Develop the different Polaroid films for the time indicated by the manufacturer (*see* **Note 11**). Spread the Polaroid print coater on the surface of the 665 positive immediately after development. (The positive prints of the 667 and 669 films do not require coating.) The 665 negatives can be stored temporary in water but, before definitive storage, immerse the negatives in 18% sodium sulfite and agitate gently for about 1 min, wash in running water for 5 min, dip in a solution of wetting agent (about 5 s), and finally dry in air (*see* **Note 12**).
13. Scan the photographic negatives to determine the intensity of the Nile red stained protein bands if a quantitative analysis is required. The quantitative analysis can also be performed directly from the image obtained with a CCD documentation system (*see* **Note 13**).

4. Notes

1. Isoelectric focusing gels do not contain SDS and should be treated with this detergent after the electrophoretic run to generate the hydrophobic SDS–protein complexes specifically stained by Nile red ***(6)***. In general, for systems

without SDS we recommend an extensive gel washing (20 min in the case of 0.75-mm thick isoelectric focusing gels) with 0.05% SDS, 0.025 *M* Tris, 0.192 *M* glycine, pH 8.3, after electrophoresis. The gel equilibrated in this buffer can be stained with Nile red following **steps 6–9** of **Subheading 3.**

2. To reduce the background fluorescence after the staining with Nile red it is necessary to preclude the formation of pure SDS micelles in the gel (*see* **Subheading 1.**). Thus, use 0.05% SDS to prepare both the separating and stacking gels and the electrophoresis buffer. This concentration is lower than the critical micelle concentration of this detergent (~0.1% ***[3]***), but is high enough to allow the formation of the normal SDS–protein complexes that are specifically stained by Nile red ***(4)***.
3. Use 2% SDS in the sample buffer to be sure that all protein samples are completely saturated with SDS. Lower concentrations of SDS in the sample buffer can produce only a partial saturation of proteins (in particular in highly concentrated samples), and consequently the electrophoretic bands could have anomalous electrophoretic mobilities. The excess SDS (uncomplexed by proteins) present in the sample buffer migrates faster than the proteins and form a broad band at the bottom of the gel (*see* **Note 5** and **ref.** *4*).
4. Storage of protein samples prepared as indicated in **Subheading 3.2.** at 4°C (or at lower temperatures) causes the precipitation of the SDS present in the solution. These samples should be incubated in a boiling-water bath to redissolve SDS before using them for electrophoresis.
5. The bottom part of the gel (i.e., from about 0.5 cm above the bromophenol blue band to the end) should be excised before the staining of the gel. Otherwise, after the addition of Nile red, the lower part of the gel produces a broad band with intense fluorescence. This band is presumably caused by the association of Nile red with the excess SDS used in the sample buffer (*see* **Note 3**). In the case of long runs, bromophenol blue and the excess SDS band diffuse into the buffer of the lower reservoir and it is not necessary to excise the gel bottom.
6. Nile red is very stable when dissolved in DMSO (*see* **Subheading 2.1.**), but this dye precipitates in aqueous solutions. Since the precipitation of Nile red in water is a rapid process and this dye is only active for the staining of SDS–protein bands before it is completely precipitated ***(4,5)***, to obtain satisfactory results, it is necessary: (a) to perform all the agitations indicated in these steps (in order to favor as much as possible the dispersion of the dye); (b) to work very rapidly in the **steps 6–9** of **Subheading 3.** Furthermore, to obtain a homogeneous staining the gel has to be completely covered with the staining solution.
7. The staining time is the same for large and small gels.
8. After the water rinsing indicated in the **step 9** of **Subheading 3.**, the staining process is completely finished and the gel can be photographed immedi-

ately. It is not necessary, however, to examine and photograph the gel just after staining. Nile red stained bands are stable and the gel can be kept in the plastic box immersed in water for 1–2 h before photography.

9. About 100 ng of protein per band can be seen by naked eye (when using the green-light transilluminator it is necessary to place a Wratten filter number 26 in front of the eyes to visualize the bands). Faint bands that are not visible by direct observation of the transilluminated gel can be clearly seen in the photographic image. Very faint bands (containing as little as 10 ng of protein) can be detected by long exposure using Polaroid film. Long integration times with a CCD camera allow the detection of 5 ng of protein per band (using both UV and green-light transilluminators). To obtain this high sensitivity it is necessary to have sharp bands; broad bands reduce considerably the sensitivity.
10. Nile red is sensitive to intense UV irradiation ***(3)***, but has a photochemical stability high enough to allow gel staining without being necessary to introduce complex precautions in the protocol (*see* **Subheading 3., steps 6–9**). For long-term storage, solutions containing this dye are kept in the dark. Transillumination of the gel for more than a few minutes produces a significant loss of fluorescence intensity. Thus, transillumination time must be reduced as much as possible both during visualization and photography. The relative short exposure times (2–12 s) required for the Polaroid film 667 and the CCD documentation system allow to make several photographs with different exposure times if necessary. With films (Polaroid 665 and 669) requiring longer exposures (4–5 min) only the first photograph from each gel shows the maximum intensity in the fluorescent bands.
11. The development time of Polaroid films is dependent on the film temperature. Store the films at 4°C (*see* **Subheading 2., item 10**), but allow them to equilibrate at room temperature before use.
12. The relatively large area (7.3 × 9.5 cm) of the Polaroid 665 negative is very convenient for further densitometric measurements (*see* **Note 13**). Furthermore, this negative can be used for making prints and digital images with an adequate level of contrast (*see*, e.g., the photograph presented in **Fig. 2**). The images obtained with the CCD documentation system can be stored in the computer and printed using different printers (inkjet printers with glossy paper yield photo quality images).
13. In quantitative analyses care has to be taken to ensure that the photographic film or the CCD documentation system have a linear response for the amounts of protein under study. Use different amounts (in the same range as the analyzed protein) of an internal standard to obtain an exposure time producing a linear response. Furthermore, the internal standard is necessary to normalize the results obtained with different gels, under different electrophoretic conditions and with different exposure and development times.

Nile red can be considered as a general stain for proteins separated in SDS gels *(4)*. However, proteins with prosthetic groups such as catalase, and proteins having anomalous SDS binding properties such as histone H5, show atypical values of fluorescence intensity after staining with Nile red *(4)*.

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Zn^{2+}-Reverse Staining Technique

Carlos Fernandez-Patron

1. Introduction

With the advent of "proteomics," many previously unidentified proteins will be isolated in gels for subsequent structural and functional characterization. It is, therefore, important that methods are available for detecting these proteins with minimal risk of modification. This chapter describes a "reverse" staining technique that facilitates the sensitive detection of unmodified proteins ***(1–9).***

The Zn^{2+} reverse staining technique exploits the ability of biopolymers (in particular, proteins and protein-SDS complexes) to bind Zn^{2+} ***(9–11)*** and that of imidazole (ImH, $C_3N_3H_{4,}$ *see* **Note 1**) to react with unbound Zn^{2+} to produce insoluble zinc imidazolate ($ZnIm_2$). Deposition of $ZnIm_2$ along the gel surface results in the formation of a deep white stained background, against which unstained biopolymer bands contrast (as biopolymer bands bind Zn^{2+}, they locally inhibit deposition of $ZnIm_2$ and are not stained; *see* **Notes 2** and **3**).

With regard to protein analysis, reverse staining is a very gentle method of detection that does not require strong acid solutions, organic dyes, chemical modifiers or protein sensitizers. Protein interactions with Zn^{2+}, established during the reverse staining step, are abrogated by metal chelation. As such, the risk of protein modification during reverse staining is minimal and reverse stained proteins can be efficiently eluted and used in biological and enzymatic assays (*see* **Note 4** and **refs.** ***7–9, 15***). Proteins can also be processed for microsequencing or mass spectrometric analysis at any time after detection (*see* **Note 5** and **refs.** ***5–9, 13, 14***).

Another benefit of the reverse staining technique is its high sensitivity of detection (~ 10 ng protein / band in SDS-PAGE gels), which is greater than that of the Coomassie blue stain (~100 ng protein / band) and approaches the

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sensitivity of the silver stains (1–10 ng protein / band). Indeed, reverse staining often reveals many proteins that display low affinity for Coomassie blue and are thus not detected with this stain (*see* **Note 6** and **ref. *11***).

Other advantages of the reverse staining technique include the speed of procedure, as it consumes ~15 min, thus being significantly faster than the Coomassie blue (> 1 hr) and silver (> 2 hr) stains. The reverse stained gels can be kept in water for several hours to years without loss of image or sensitivity of detection. Similar to other stains, reverse stained patterns can be analyzed densitometrically, and a gel "toning" procedure has been recently developed to preserve the image upon gel drying ***(12).***

The Zn^{2+} reverse staining technique can be applied to detect virtually any gel-separated biopolymer that binds Zn^{2+} (i.e., proteins/peptides, glycolipids, oligonucleotides, and their multimolecular complexes) (**ref. *9*** and references therein). Moreover, these biopolymers are detected regardless of the electrophoresis system used for their separation (*see* **Notes 7, 8,** and **9** and **ref. *9***), which was not possible with the previously developed metal salt stains ***(1–3)***. Therefore, Zn^{2+} reverse staining is a widely applicable detection method.

The SDS-PAGE method of protein electrophoresis is probably the most popular. Thus, the author chose to describe a standard reverse staining method that works very well with SDS-PAGE. The detection of proteins, peptides and their complexes with glycolipids in less commonly used gel electrophoresis systems is addressed as well (*see* **Notes 7** to **9**).

2. Materials

Reagent- and analytical-grade zinc sulfate, imidazole, acetic acid, sodium carbonate are obtained from Sigma (St. Louis, MO).

1. Equilibration solution (1x): 0.2 M imidazole, 0.1% (w/v) SDS (*see* **Note 1**).
2. Developer (1x): 0.3 M zinc sulfate.
3. Storage solution for reverse stained gels (1x): 0.5 % (w/v) sodium carbonate.

All solutions are prepared as 10 times concentrated stocks, stored at room temperature, and diluted (1:10) in distilled water, to yield the working concentration (1x), just before use.

3. Methods

3.1. Polyacrylamide gel electrophoresis

SDS-PAGE is conducted following the method of Laemmli ***(16)***. Native PAGE is conducted following the protocol of Laemmli, except that SDS is not included in the gel and electrophoresis solutions. Conventional agarose gel electrophoresis is conducted in 0.8% agarose gels and using Tris-acetate, pH 8.0; as gel and running buffer ***(17)***.

3.2. Standard Reverse Staining of SDS-PAGE and Native PAGE Gels

The following reverse staining method detects proteins in standard polyacrylamide gels (*see* **Note 2, Fig. 1**). All incubations are performed under continuous gentle agitation in a plastic or glass tray with a transparent bottom. The volume of the corresponding staining/storage solutions must be enough to cover the gel (typically 50 ml for 1 minigel (10 cm × 7 cm × 0.75 mm)).

1. Following electrophoresis, the gel is incubated for 15 min in the equilibration solution (*see* **Note 2**).
2. To develop the electropherogram, the imidazole solution is discarded and the gel soaked for 30–40 s in developer solution. **Caution!:** This step must not be extended longer than 45 s or band overstaining and loss of the image will occur. Overstaining is prevented by pouring off the developer solution and rinsing the gel 3–5 times (1 min) in excess water (*see* **Note 3**).

At this point, the reverse stained gel can be photographed **(Fig. 1).** Photographic recording is best conducted with the gel placed on a glass plate held a few centimeters above a black underground and under lateral illumination.

While SDS-PAGE is a popular, high-resolution method for separating complex protein mixtures, sometimes it is desired to avoid either protein denaturation or disruption of macromolecular complexes during electrophoresis. In this case, the proteins are separated in the absence of SDS (native PAGE or agarose gel electrophoresis). Therefore, it is desirable to avoid the use of SDS during the reverse

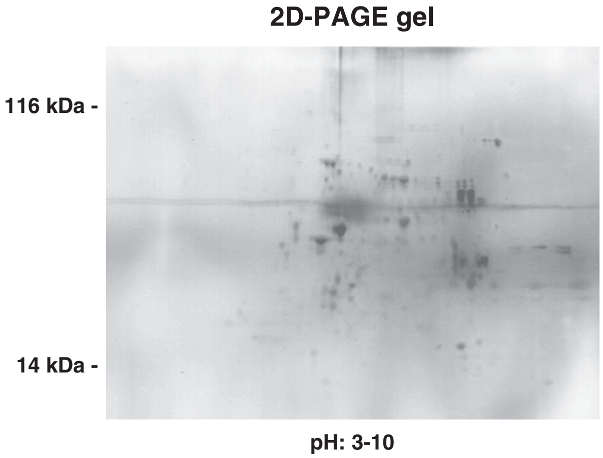

Fig. 1. Reverse staining of rat brain homogenate proteins (70 µg load) after 2D-PAGE. Proteins in a wide range of molecular weights and isoelectric points are detected as transparent spots. These spots contain protein-SDS complexes that bind Zn^{2+} and thereby inhibit the precipitation of ZnIm$_2$ locally.

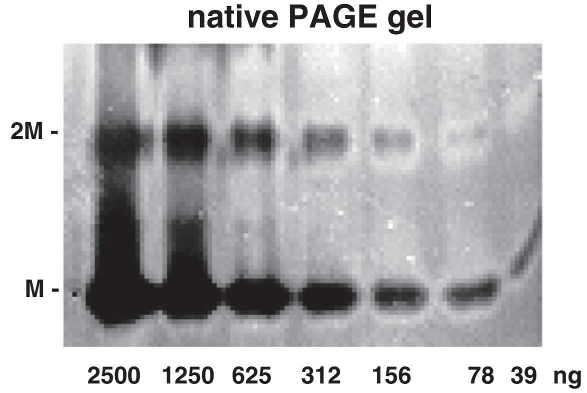
native PAGE gel
2M -
M -
2500 1250 625 312 156 78 39 ng

Fig. 2. Reverse staining of serial dilutions of human serum albumin (HSA) after native PAGE. HSA migrates yielding two main bands corresponding to its monomer (M) and its dimer (2 M) and is detected under native conditions (no SDS) due to its natural ability to complex with Zn^{2+}.

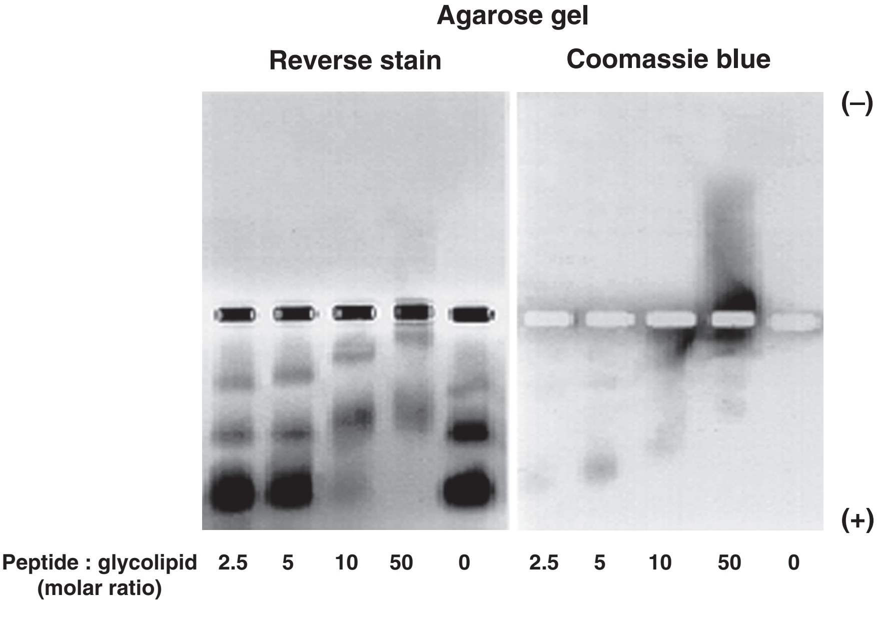
Agarose gel
Reverse stain
Coomassie blue
(–)
(+)
Peptide : glycolipid (molar ratio)
2.5 5 10 50 0
2.5 5 10 50 0

Fig. 3. Reverse staining of complexes between a synthetic cationic peptide with lipopolysaccharide binding properties and the glycolipid of a *N. meningitidis* strain. Formation of complexes was promoted by incubating (37°C) peptide and glycolipid for 30 min in 25 m*M* Tris-HCl, 0.1% Triton X-100, pH 8.0; at indicated peptide:glycolipid molar ratios. Reverse staining revealed bands of both: peptide.glycolipid complexes and uncomplexed glycolipid. Coomassie blue stained mainly the uncomplexed peptide migrating towards the negative (–) electrode. Coomassie blue failed to stain the glycolipid bands and stained peptide.glycolipid complexes very weakly.

staining step. Two procedures have been developed to resolve this problem (*see* **Notes 8** and **9**, **Figs. 2** and **3**).

4. Notes

1. Imidazole is a five-membered heterocyclic ring containing a tertiary ("pyridine") nitrogen at position 3, and a secondary ("pyrrole") nitrogen at position 1. It is a monoacidic base whose basic nature is due to the ability of pyridine nitrogen to accept a proton. The pyrrole nitrogen can lose the hydrogen atom producing imidazolate anion (Im^-), at high pH values (pK_a ~14.2). However, deprotonation of imidazole's pyrrolic nitrogen may also occur at lower pH values, upon complexation of Zn^{2+} at the pyridinium nitrogen (**ref.** ***9*** and references therein). As a result, $ZnIm_2$ can form and precipitate at pH > 6.2. Upon treatment of a polyacrylamide or agarose electrophoresis gel with salts of zinc(II) and imidazole, a complex system is generated. Complexity is due to the presence of amide groups in the polyacrylamide matrix and sulfate groups in the agarose gel as well as Tris, glycine and dodecylsulfate, hydroxyle and carbonate anions in the electrophoresis buffers. These groups can coordinate with Zn(II), act as counteranions in the complexes of zinc with imidazole, and lead to the formation of complex salts and hydroxides. Diffusion phenomena (reflected in the times required for optimal gel "equilibration" and "development" during reverse staining) also critically influence the reverse staining reactions between Zn^{2+} and imidazole (*see* **Notes 2** and **3**). Nevertheless, when the protocol described in this chapter is followed, $ZnIm_2$ is the major component of the precipitate that stains the gels treated with zinc sulfate and imidazole ***(9)***.
2. This reverse staining protocol is optimized for use with any standard PAGE system regardless of whether PAGE is the first (1D-PAGE) or the second (2D-PAGE) dimension in the separation strategy. The equilibration step assures that proteins in the gel are all uniformly coated with SDS. Therefore, protein's ability to bind Zn^{2+} is modulated by the protein-SDS complex and limits of detection (10 ng protein / band) are similar for gels cast with and without SDS (i.e., SDS-PAGE and native PAGE, respectively). The larger the gel thickness or acrylamide concentration, the longer the equilibration step. A 15 min long equilibration is enough for gels with ≤ 15% acrylamide and ≤ 1 mm thickness. Preparative gels are often as thick as 3–5 mm; these gels should be equilibrated for 30–60 min. Insufficient equilibration may result in faint reverse stained patterns, which may fade upon prolonged storage.
3. Development time must be between 30 and 45 s as insufficient development results in pale background staining and over-development causes overstaining. An overstained gel can be re-stained. For this, the gel is treated with 10 mM EDTA or 100 m*M* glycine for 5–10 min to redissolve the

white $ZnIm_2$ precipitate that has deposited on the gel surface, rinsed in water (30 s) and, finally, soaked in the "storage" sodium carbonate solution. If the reverse stained pattern does not restore in ~5 min, the reverse staining procedure can be repeated as indicated in Methods. Usually, the reverse stained pattern will restore during the equilibration step due to the precipitation of traces of Zn^{2+} already present in the gel with the imidazole from the equilibration solution. If the above suggestions do not lead to a homogenous reverse stained pattern of suitable quality, the gel can be "positively" restained with Coomassie blue or silver ***(5).*** In this case, it is recommended that the reverse-stained gel be treated with EDTA (50 mM, pH 8.0; 30 min incubation) to free proteins of Zn^{2+}. Then, the gel can be processed with Coomassie blue or silver stains.

4. Protein elution from reverse stained gels can be performed following any conventional method such as electroelution or passive elution; however, a highly efficient procedure has been developed ***(7, 8).*** The reverse stained band of interest is excised, placed in a (1 ml) plastic vial and incubated with EDTA (50 m*M*, 2 × 5 min and 10 m*M*, 1 × 5 min) to chelate protein bound zinc ions. Supplementation of EDTA with non-ionic detergents is optional but convenient if protein is to be in-gel refolded; e.g., Triton X-100 was useful when refolding proteins that were to be bioassayed ***(9, 15).*** EDTA (or EDTA, detergent mixture) is replaced by an appropriate "assay" buffer (e.g., phosphate saline solution), in which the protein band is equilibrated. Finally, the band is homogenized and protein is eluted into an appropriate volume of the assay buffer ***(7, 8, 14).*** The slurry is centrifuged and the supernatant filtered to collect a clean, transparent solution of protein ready for subsequent analysis ***(7–9, 14, 15).***
5. An important application of reverse staining is in structural/functional proteomics ***(13, 14).*** Identification of proteins separated by electrophoresis is a prerequisite to the construction of protein databases in proteome projects. Matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) has a sensitivity for peptide detection in the lower fmol range. In principle, this sensitivity should be sufficient for an analysis of small amounts of proteins in silver-stained gels. However, a variety of known factors (e.g., chemical sensitizers such as glutaraldehyde) and other unknown factors modify the silver-stained proteins, leading to low sequence coverage ***(13).*** Low-abundance proteins have been successfully identified after $ZnIm_2$-reverse-staining ***(13, 14).***
6. Reverse staining of gels that have been already stained with Coomassie blue reveals proteins undetected with Coomassie blue, thus improving detection. Double staining can enhance sensitivity of detection in silver-stained gels as well. Before subjecting a Coomassie (or silver)-stained

gel to double staining, the gel should be rinsed in water (2–3 × 10 min). This step assures a substantial removal of acetic acid from Coomassie and silver stains. Then, the gel is subjected to the standard reverse staining protocol. The resultant double stained patterns consist of the previously seen Coomassie blue (brown, in the case of silver)–stained bands and new unstained (reverse-stained) bands that contrast against the deep-white, $ZnIm_2$, stained background.

7. Reverse staining can be used in combination with other "specific" protein stains to indicate the presence of both protein and glycolipid. As with proteins, the biological properties of the presumed glycolipid bands are testable in a functional experiment following elution from the reverse stained gel ***(9, 18)***. After the electrophoresis step, the gel is rinsed for 30 min (2x, 15 min) in aqueous solution of methanol (40%, v/v). This step presumably removes excess SDS from glycolipid molecules that co-migrate with the proteins in SDS-PAGE, making the glycolipid adopt a conformation with high affinity for Zn^{2+}. Next, the gel is reverse stained (Methods). Similar to proteins, glycolipid bands show up transparent and unstained. Sensitivity of detection is ~10 ng glycolipid / band. If the same (or a parallel gel) is treated with Coomassie blue (silver) stain, that does not detect glycolipids; protein and glycolipid bands can be distinguished by their distinct staining and migration properties ***(9, 18)***. A replicate protein sample treated with proteinase K is a useful control, as this protease digests protein, but not glycolipid.
8. An alternative method of precipitating $ZnIm_2$ facilitates the reverse staining of native PAGE gels without the use of SDS ***(9)***. A *quick* and a *slow* reverse staining procedure were implemented ***(9)***. The *quick* version makes use of the characteristic neutral to basic pH of the gels immediately after electrophoresis. When the gel is incubated (3–6 min) in an slightly acidic solution containing zinc sulfate and imidazole (pre-mixed to yield the molar ratio Zn(II) : ImH = 15 m*M* : 30 m*M*, pH 5.0 – 5.5), the gel stains negatively as $ZnIm_2$ deposits along its surface. Blotchy deposits are prevented by intense agitation and resolubilized by lowering the pH of the zinc-imidazole solution; this also provides a basis for a *slow* version of this method. In the slow version **(Fig. 2)**, the gel is incubated (10–20 min) in a solution of zinc sulfate (15 m*M*) and imidazole (30 m*M*) adjusted to pH 4.0. No precipitation occurs at this pH. The solution is poured off, the gel is rinsed with water (30 s); the electropherogram is developed by incubating the gel during ~5 min in 1% sodium carbonate. Sodium carbonate increases the pH first at the gel surface. Therefore, Zn^{2+} and imidazole, already present in the gel, react at the gel surface to form $ZnIm_2$. Again, protein bands are not stained as they complex with Zn^{2+} and locally prevent the precipitation of $ZnIm_2$.

9. Many proteins and peptides as well as glycolipids and their complexes with certain proteins/peptides separate well on agarose gels under nondenaturing conditions ***(9).*** To reverse stain an agarose gel ***(9)***; following electrophoresis, the gel is incubated for 25 – 30 min in a zinc sulfate-imidazole solution (Zn(II) : ImH = 15 mM: 30 mM, adjusted to pH 5.0 with glacial acetic acid). During this step, Zn^{2+} and imidazole diffuse into agarose matrix. The gel is then rinsed in water for 5–8 min to remove any excess of the staining reagents from the gel surface. The reverse stained electropherogram is developed by incubating the gel for 5–8 minutes in 1% Na_2CO_3 **(Fig. 3).** Of note, due to poorly understood factors, agarose gels are not as amenable to reverse staining as polyacrylamide gels. Patterns of positive and negative bands are often seen in agarose gels.

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Protein Staining with Calconcarboxylic Acid in Polyacrylamide Gels

Wei-Tao Cong, Sun-Young Hwang, Li-Tai Jin, and Jung-Kap Choi

1. Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has become a highly reliable separation technique for protein characterization. Broad application of electrophoresis techniques has required the development of detection methods that can be used to visualize the proteins separated on polyacrylamide gels. A few of these methods are Coomassie blue (CB) staining ***(1)***, silver staining ***(2)***, fluorescent staining ***(3–5)***, specific enzyme visualization ***(6)***, and radioactive detection ***(7)***.

CB staining is the most commonly used method owing to its proven reliability, simplicity, and economy (***8, 9***), but it lacks sensitivity compared with the silver-staining method. In addition, the staining/destaining process is time consuming ***(10)***. Silver staining is the most sensitive nonradioactive protein detection method currently available, and can detect as little as 10 fg of protein (***11, 12***). However, it has several drawbacks, such as high-background staining, multiple steps, high cost of reagent, and toxicity of formaldehyde ***(10)***.

In this chapter a protein staining method using calconcarboxylic acid (1-[2-hydroxy-4-sulfo-l-naphthylazo]-2-hydroxy-3-naphthoic acid) is described (*see* **Note 1; Fig. 1; ref.** ***13***). This method can be performed by both simultaneous and postelectrophoretic staining techniques. Simultaneous staining using 0.01% of NN in upper reservoir buffer eliminates the poststaining step, and thus enables detection of the proteins more rapidly and simply. In poststaining, proteins can be stained by a 30 min incubation of a gel in 40% methanol/7% acetic acid solution of 0.05% NN and destaining in 40% methanol/7% acetic acid for 40 min with agitation. NN staining can detect as little as 10 ng of bovine serum albumin (BSA) by poststaining and 25 ng by simultaneous staining, compared to 100 ng detectable by CB poststaining (*see* **Note 2**). These techniques produce

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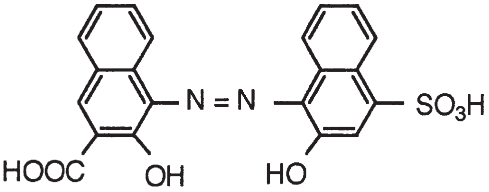

Fig. 1. Structure of NN.

protein-staining patterns identical to the ones obtained by the conventional CB staining and also work well in nondenaturing PAGE, like in SDS-PAGE. The bands stained with NN present purple color. In addition, NN staining gives better linearity than CB staining, although the slopes (band intensity/amount of protein) of it are somewhat lower (*see* **Table 1**, **Fig. 2**). It suggests that NN staining is more useful than CB staining for quantitative work of proteins.

2. Materials

1. All working solutions should be freshly prepared with distilled water and clean glassware or plastic ware. All steps were carried out at room temperature with shaking.
2. Destaining solution (1.0 L): Mix 530 mL distilled water with 400 mL methanol and 70 mL glacial acetic acid.
3. Simultaneous staining solution (1.0% [w/v] NN): Dissolve 1.0 g NN (pure *NN without K_2SO_4) in 100 mL reservoir buffer. Stir until fully dissolved at 50–60°C (stable for months at 4°C) (*see* **Note 3**).
4. Poststaining solution (0.05% [w/v] NN): Dissolve 0.05 g NN (pure *NN) in 100 mL destaining solution. Stir until dissolved thoroughly (store at room temperature).

3. Methods

3.1. Simultaneous Staining

The method is based on the procedure of Borejdo and Flynn ***(14)***, and is described for staining proteins in a 7.5% SDS-polyacrylamide gel.

1. Electrophorese the samples for 10 min to allow protein penetration into the upper gel phase.
2. Turn off the power, and then add 1% NN dissolved in reservoir buffer to the upper reservoir buffer to give a final concentration of 0.01–0.015% NN (*see* **Notes 3** and **4**).
3. Stir the reservoir buffer sufficiently to ensure homogeneity.
4. Resume electrophoresis.
5. Immediately after electrophoresis, remove the stained gel from the apparatus.
6. Destain the stained gels in 40% methanol/7% acetic acid for 30 min. To destain completely, change destaining solution several times and agitate (*see* **Notes 5–7**).

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*NN diluted with 100- to 200-fold K_2SO_4 has been used as an indicator for the determination of calcium in the presence of magnesium with EDTA.

Table 1
Linearity of CB and NN Staining for Four Purified Proteins

	Slope[b]		y-intercept[b]		Correlation coefficient[b]	
Protein[a]	A	B	A	B	A	B
BSA	14.1	10.1	13.2	6.4	0.986	0.994
OVA	8.7	7.1	2.7	1.4	0.993	0.998
G-3-P DHase	8.0	5.8	5.3	2.2	0.997	0.999
CA	15.4	13.5	12.5	8.8	0.986	0.997

[a] Proteins were separated on 12.5% polyacrylamide gel, densities and band area were determined with computerized densitometer. Some of the data are illustrated in **Fig. 2**. The range of amount of proteins was 0.25–12.5 µg. The number of points measured was six (0.25, 0.5, 1.0, 2.5, 5.0, and 12.5 µg).

[b] Slopes, *y*-intercepts, and correlation coefficients were determined by linear regression analysis. A, CB staining; B, NN staining.

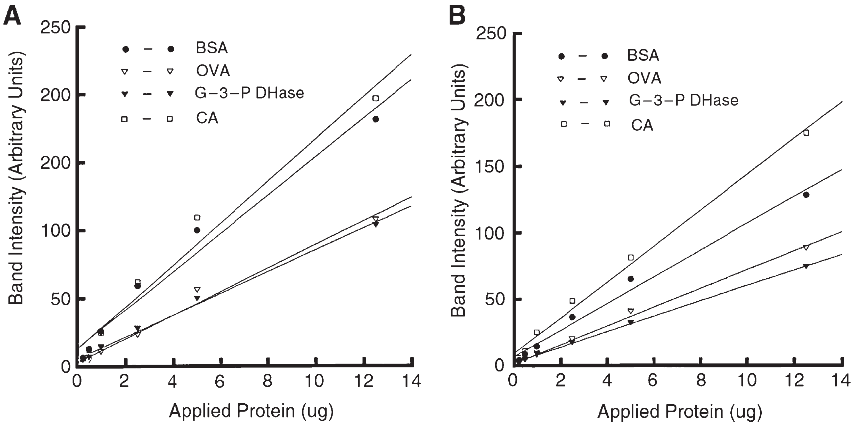

Fig. 2. Densitometric comparison of CB and NN staining. Proteins were separated on 12.5% gels. (A) poststaining with 0.1% CB; (B) simultaneous staining with 0.015% NN. Densitometric scanning was performed at 585 nm (CB) and 580 nm (NN). The curves are fitted by the method of least squares. Each point represents the mean of three determinations. BSA, bovine serum albumin; OVA, ovalbumin; G-3-P DHase, glyceraldehyde-3-phosphate dehydrogenase; CA, carbonic anhydrase

3.2. Postelectrophoretic Staining

1. Agitate the freshly run gel in 0.05% NN staining solution for 30 min.
2. Pour off the staining solution and rinse the gel with changes of destaining solution (two to three times). Staining solution can be reused several times.
3. Destain the stained gels in 40% methanol/7% acetic acid for 40 min with agitation (*see* **Notes 8** and **9**).

4. Notes

1. NN has several functional groups, such as hydroxyl, diazoic, carboxyl, and sulfonate groups (*see* **Fig. 1**). At acidic pH, NN probably forms electrostatic bonds with protonated amino groups, which are stabilized by hydrogen bonds and Van der Waals forces, as CB does *(1)*.
2. The protocol for poststaining is the same as that for CB staining, except for staining/destaining times and dye used.
3. The preparation of staining solution requires stirring and warming at 50–60°C since NN is poorly soluble.
4. The simultaneous staining method allows one to control the intensity of stained bands reproducibly by adjusting the concentration of the dye in the upper reservoir. More than 0.02% NN in the upper reservoir buffer does not increase sensitivity but requires more destaining time.
5. Gel staining/destaining with NN is pH dependent. Intense staining occurs at pH 1.6–4.4, and weak staining with blue-purple color is observed at alkaline pH. In excessively strong acidic solution (pH<1.0), however, the staining effect is markedly decreased, because the solubility of the dye is decreased and, thus replacement between dye anions and acetate ions may be suppressed (*see* **Fig. 3**). In destaining solution, the stained band diffuses, and its intensity is decreased significantly at pH higher than 4.4. Destaining in this pH range is rather slow compared with that in strongly acidic conditions.
6. The rate of destaining speeds up with increasing methanol content; however, at high methanol content (>55%), gels are opaqued and shrunken. Addtionally, increasing the temperature of destaining solution is a great help in removing background (at 60–70°C, in 5 min), although sensitivity is a little reduced.

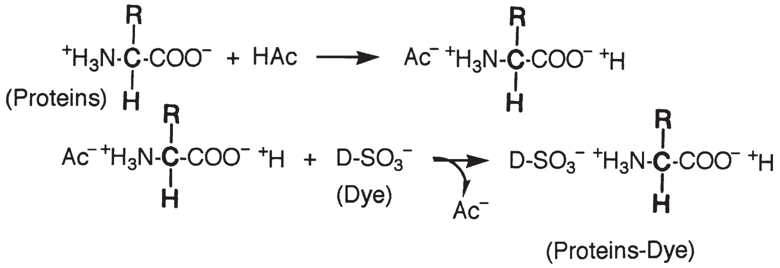

Fig. 3. Mechanism of protein–dye interaction in acidic solution.

7. Destaining can be completed within 30 min in 7.5% polyacrylamide gels, but destaining time should be increased for 10 and 12.5% gels (50–60 min).
8. Bands stained with NN are indefinitely stable when gels are stored in a refrigerator wrapped up in polyethylene film or dried on Whatmann No. 1 filter paper.
9. Throughout the staining/destaining processes, it is necessary to agitate the gel container using a shaker.

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Detection of Proteins in Polyacrylamide Gels by Silver Staining

Michael J. Dunn

1. Introduction

The versatility and resolving capacity of polyacrylamide gel electrophoresis has resulted in this group of methods becoming the most popular for the analysis of patterns of protein expression in a wide variety of complex systems. These techniques are often used to characterise protein purity and to monitor the various steps in a protein purification process. Moreover, two-dimensional polyacrylamide gel electrophoresis (2-DE) remains the core technology of choice for separating complex protein mixtures in the majority of proteome projects ***(1).*** This is due to its ability to separate simultaneously thousands of proteins and to indicate post-translational modifications that result in alterations in protein pI or M_r. Additional advantages are the high-sensitivity visualization of the resulting 2-DE separations, compatibility with quantitative computer analysis to detect differentially regulated proteins ***(2),*** and the relative ease with which proteins from 2-DE gels can be identified and characterised by mass spectrometry (MS) ***(3).***

After 2-DE, the separated proteins must be visualised at high sensitivity, and the detection method should ideally combine the properties of an extended dynamic range, a linear staining response, reproducibility, and compatibility with post-electrophoretic protein identification procedures, especially those based on the use of MS. Unfortunately, no current staining method can fulfil all of these requirements.

Staining methods based on the use of the organic dye, Coomassie Brilliant Blue (CBB), have been used extensively for the detection of proteins on 2-DE gels, due to their low price, ease of use and compatibility with downstream

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protein identification by MS. However, methods using CBB are limited by their lack of sensitivity (around 200–500 ng protein per spot), such that only a few hundred protein spots can usually be visualised on a 2-DE gel, even if milligram amounts of protein sample are loaded ***(4).*** The need to detect small amounts of protein within 2-DE gel spots has resulted in the development of a range of more sensitive detection methods ***(4).*** The first method that was able to meet this requirement and which is still used in many proteomic projects is silver staining.

The ability of silver to develop images was discovered in the mid-17th century and this property was exploited in the development of photography, followed by its use in histological procedures. Silver staining for the detection of proteins following gel electrophoresis was first reported in 1979 by Switzer et al ***(5),*** resulting in a major increase in the sensitivity of protein detection. More than 100 publications have subsequently appeared describing variations in silver staining methodology ***(4, 6).*** This group of procedures is generally accepted to be around 100 times more sensitive than methods using CBB R-250, being able to detect low ng amounts of protein per gel band or spot.

All silver staining procedures depend on the reduction of ionic silver to its metallic form, but the precise mechanism involved in the staining of proteins has not been fully established. It has been proposed that silver cations complex with protein amino groups, particularly the ε-amino group of lysine ***(7),*** and with sulphur residues of cysteine and methionine ***(8).*** However, Gersten and his colleagues have shown that “stainability” cannot be attributed entirely to specific amino acids and have suggested that some element of protein structure, higher than amino acid composition, is responsible for differential silver staining ***(9).***

Silver staining procedures can be grouped into two types of method depending on the chemical state of the silver ion when used for impregnating the gel. The first group is alkaline methods based on the use of an ammoniacal silver or diamine solution, prepared by adding silver nitrate to a sodium-ammonium hydroxide mixture. Copper can be included in these diamine procedures to give increased sensitivity, possibly by a mechanism similar to that of the Biuret reaction. The silver ions complexed to proteins within the gel are subsequently developed by reduction to metallic silver with formaldehyde in an acidified environment, usually using citric acid. In the second group of methods, silver nitrate in a weakly acidic (approximately pH 6) solution is used for gel impregnation. Development is subsequently achieved by the selective reduction of ionic silver to metallic silver by formaldehyde made alkaline with either sodium carbonate or NaOH. Any free silver nitrate must be washed out of the gel prior to development, as precipitation of silver oxide will result in high background staining.

Silver stains are normally monochromatic, resulting in a dark brown image. However, if the development time is extended, dense protein zones become saturated and colour effects can be produced. Some staining methods have been designed to enhance these colour effects, which were claimed to be related to the nature of the polypeptides detected ***(10).*** However, it has now been established that the colours produced depend on: (a) the size of the silver particles, (b) the distribution of silver particles within the gel, and (c) the refractive index of the gel ***(11).***

Rabilloud has compared several staining methods based on both the silver diamine and silver nitrate types of procedure ***(12).*** The most rapid procedures were found to be generally less sensitive than the more time-consuming methods. Methods using glutaraldehyde pretreatment of the gel and silver diamine complex as the silvering agent were found to be the most sensitive. However, it should be noted that the glutaraldehyde and formaldehyde present in many silver staining procedures results in alkylation of α- and ε-amino groups of proteins, thereby interfering with their subsequent chemical characterisation by MS. To overcome this problem, silver staining protocols compatible with mass spectrometry in which glutaraldehyde is omitted have been developed ***(13, 14)*** but these suffer from a decrease in sensitivity of staining and a tendency to a higher background. This problem can be overcome using post-electrophoretic fluorescent staining techniques ***(4, 15).*** One the most commonly used stains of this type is SYPRO Ruby (*see* Chapter 50), which has a sensitivity approaching that of standard silver staining and is fully compatible with protein characterisation by MS ***(16).***

The method of silver staining we describe here is recommended for analytical applications and is based on that of Hochstrasser et al ***(17, 18)*****,** together with modifications and technical advice that will enable an experimenter to optimise results. An example of a one-dimensional SDS-PAGE separation of the total proteins of human heart proteins stained by this procedure is shown in **Fig. 1.** The power of 2-DE combined with sensitive detection by silver staining to display the complex protein profile of a whole tissue lysate is shown in **Fig. 2.**

2. Materials

1. All solutions should be freshly prepared, and overnight storage is not recommended. Solutions must be prepared using clean glassware and deionised, distilled water.
2. Gel fixation solution: Trichloroacetic acid (TCA) solution, 20% (w/v).
3. Sensitisation solution: 10% (w/v) glutaraldehyde solution.

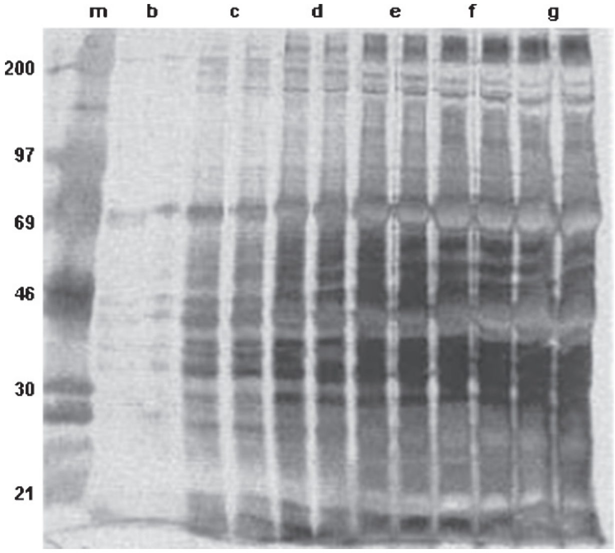

Fig. 1. One-dimensional 12%T SDS-PAGE separation of human heart proteins (lanes b-g) visualised by silver staining. Lane (m) contains the M_r marker proteins and the scale at the left indicates $M_r \times 10^{-3}$. The sample protein loadings were (b) 1 µg, (c) 5 µg, (d) 10 µg, (e) 25 µg, (f) 50 µg, (g) 100 µg.

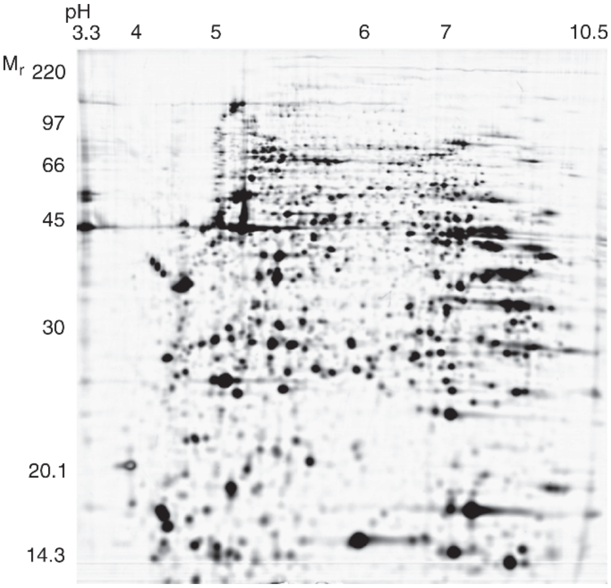

Fig. 2. 2-DE separation of human heart proteins visualised by silver staining. A loading of 100 µg protein was used. The first dimension was pH 3–10 NL IPG IEF and the second dimension was 12% T SDS-PAGE. The scale at the top indicates the non-linear pH gradient used in the first IPG 3–10 NL IEF dimension, while the scale at the left indicates $M_r \times 10^{-3}$.

4. Silver diamine solution: 21 mL of 0.36% (w/v) NaOH is added to 1.4 mL of 35% (w/v) ammonia and then 4 mL of 20% (w/v) silver nitrate is added drop-wise with stirring. When the mixture fails to clear with the formation of a brown precipitate, further addition of a minimum amount of ammonia results in the dissolution of the precipitate. The solution is made up to 100 mL with water. The silver diamine solution is unstable and should be used within 5 min.
5. Developing solution: 2.5 mL of 1% (w/v) citric acid, 0.26 mL of 36% (w/v) formaldehyde made up to 500 mL with water.
6. Stopping solution: 40% (v/v) ethanol, 10% (v/v) acetic acid in water.
7. Farmer's reducer: 0.3% (w/v) potassium ferricyanide, 0.6% (w/v) sodium thiosulphate, 0.1% (w/v) sodium carbonate.

3. Method

Note: *All incubations are carried out at room temperature with gentle agitation*

3.1. Fixation

1. After electrophoresis, fix the gel immediately (*see* **Note 1**) in 200 mL (*see* **Note 2**) of TCA (*see* **Note 3**) for a minimum of 1 h at room temperature. High-percentage polyacrylamide and thick gels require an increased period for fixation, and overnight soaking is recommended.
2. Place the gel in 200 mL of 40% (v/v) ethanol, 10% (v/v) acetic acid in water and soak for 2 × 30 min (*see* **Note 4**).
3. Wash the gel in excess water for 2 × 20 min, facilitating the rehydration of the gel and the removal of methanol. An indication of rehydration is the loss of the hydrophobic nature of the gel.

3.2. Sensitisation

1. Soak the gel in a 10% (w/v) glutaraldehyde solution for 30 min at room temperature (*see* **Note 5**).
2. Wash the gel in water for 3 × 20 min to remove excess glutaraldehyde.

3.3. Staining

1. Soak the gel in the silver diamine solution for 30 min. For thick gels (1.5 mm), it is necessary to use increased volumes so that the gels are totally immersed. Caution should be exercised in disposal of the ammoniacal silver reagent, since it decomposes on standing and may become explosive. The ammoniacal silver reagent should be treated with dilute hydrochloric acid (1 N) prior to disposal.
2. Wash the gel (3 × 5 min) in water.

3.4. Development

1. Place the gel in developing solution. Proteins are visualised as dark brown zones within 10 min (*see* **Note 6**), after which the background will gradually increase (*see* **Note 7**). It is important to note that the reaction displays inertia, and that

staining will continue for 2–4 min after removal of the gel from the developing solution. Staining times in excess of 20 min usually result in an unacceptable high background (*see* **Note 8**).

4. Terminate staining by immersing the gel in stopping solution.
5. Wash the stained gel in water prior to storage or drying.

3.5. Destaining

Partial destaining of gels using Farmer's reducing reagent is recommended for the controlled removal of background staining that obscures proper interpretation of the protein pattern.

1. Wash the stained gel in water for 5 min to remove the stop solution.
2. Place the gel in Farmer's reducer for a time dependent upon the intensity of the background.
3. Terminate destaining by returning the gel to the stop solution.

4. Notes

1. Gloves should be worn at all stages when handling gels, since silver staining will detect keratin proteins from the skin.
2. Volumes of the solutions used at all stages should be sufficient such that the gel is totally immersed. If the volume of solution is insufficient for total immersion, staining will be uneven and the gel surface can dry out.
3. A mixture of alcohol, acetic acid and water (9:9:2) is recommended for gel fixation in many published protocols, but TCA is a better general protein fixative and its use is compatible with silver staining provided that the gel is washed well after fixation to remove the acid.
4. In addition to removing TCA, the washing step also effectively removes reagents such as Tris, glycine and detergents (especially SDS) which can bind silver and result in increased background staining.
5. Treatment of the gel with reducing agents such as glutaraldehyde prior to silver impregnation results in an increase in staining sensitivity by increasing the speed of silver reduction on the proteins.
6. If image development is allowed to proceed for too long, dense protein zones will become saturated and negative staining will occur, leading to serious problems if quantitative analysis is attempted. In addition, certain proteins stain to give yellow or red zones regardless of protein concentration, and this effect has been linked to the post-translational modification of the proteins.
7. An inherent problem with the staining of gradient SDS-PAGE gels is uneven staining along the concentration gradient. The less concentrated polyacrylamide region develops background staining prior to the more concentrated region. A partial solution to this problem is to increase the time of staining in silver diamine (*see* **Subheading 3.3., step 1**).

8. Various chemicals used in one- and two-dimensional electrophoresis procedures can inhibit staining, whereas others impair resolution or produce artefacts. Acetic acid will inhibit staining and should be completely removed prior to the addition of silver diamine solution **(Subheading 3.3., step 1).** Glycerol, used to stabilise SDS gradient gels during casting, and urea, used as a denaturing agent in isoelectric focusing (IEF), are removed by water washes. Agarose, often used to embed rod IEF gels onto SDS-PAGE gels in 2-D PAGE procedures, contains peptides that are detected by silver staining as diffuse bands and give a strong background. Tris, glycine and detergents (especially SDS) present in electrophoresis buffers can complex with silver and must be washed out with water prior to staining. The use of 2-mercaptoethanol as a disulphide bond reducing agent should be avoided since it leads to the appearance of two artefactual bands at 50 and 67 kDa on the gel ***(19)***.
9. Radioactively labelled proteins can be detected by silver staining prior to autoradiography or fluorography for the majority of the commonly used isotopes (^{14}C, ^{35}S, ^{32}P, ^{125}I). In the case of ^{3}H, however, silver deposition will absorb most of the emitted radiation.

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Background-Free Protein Detection in Polyacrylamide Gels and on Electroblots Using Transition Metal Chelate Stains

Wayne F. Patton

1. Introduction

Electrophoretically separated proteins may be visualized using organic dyes such as Ponceau Red, Amido Black, Fast Green, or most commonly Coomassie Brilliant Blue ***(1,2)***. Alternatively, sensitive detection methods have been devised using metal ions and colloids of gold, silver, copper, carbon, or iron ***(3–12)***. Metal chelates form a third class of stains, consisting of transition metal complexes that bind avidly to proteins resolved in polyacrylamide gels or immobilized on solid-phase membrane supports ***(13–27)***. In recent years, metal chelate stains have been designed and optimized specifically for compatibility with commonly used microchemical characterization procedures employed in proteomics. The metal chelate stains are simple to implement, and do not contain extraneous chemicals such as glutaraldehyde, formaldehyde, or Tween-20 that are well known to interfere with many downstream protein characterization procedures.

Metal chelates can be used to detect proteins on nitrocellulose, poly(vinylidene difluoride) (PVDF), and nylon membranes as well as in polyacrylamide and agarose gels. The metal complexes do not modify proteins, and are compatible with immunoblotting, lectin blotting, mass spectrometry, and Edman-based protein sequencing ***(13–17,22–27)***. Metal chelate stains are suitable for routine protein measurement in solid-phase assays owing to the quantitative stoichiometry of complex formation with proteins and peptides ***(15,16)***. Such solid phase protein assays are more sensitive and resistant to chemical interference than their solution-based counterparts ***(15)***.

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A variety of metal ions and organic chelating agents may be combined to form metal chelate stains but only a few have been evaluated extensively for protein detection in electrophoresis. Ferrene S has been utilized for the specific detection of iron-containing proteins, such as cytochrome *c*, transferrin, ferritin, and lactoferrin in polyacrylamide gels ***(18)***. In this situation the metal complex only forms when the chelate interacts with the native metal ion bound within the protein itself. Copper phthalocyanine 4, 4' ,4", 4'" -tetrasulfonic acid has been shown to stain total protein in electrophoresis gels and on nitrocellulose membranes ***(19)***. In addition, we demonstrated the use of Ferrene S-ferrous, Ferrozine-ferrous, ferrocyanide-ferric, and Pyrogallol Red-molybdate complexes for colorimetric detection of electrophoretically separated proteins immobilized on membranes ***(13–16)***. In 1978, a pink bathophenanthroline disulfonate-ferrous complex was reported as a nonspecific protein stain for polyacrylamide gel electrophoresis ***(20)***. The stain is rather insensitive and was later modified by substituting [^{59}Fe] into the complex in order to detect proteins by autoradiography ***(21)***. Although increasing sensitivity substantially, the hazards associated with working with radioactivity and the burden of license application for an infrequently used radioisotope have precluded routine utilization of bathophenanthroline disulfonate-[^{59}Fe] as a general protein stain. Measuring light emission is intrinsically more sensitive than measuring light absorbance, as the later is limited by the molar extinction coefficient of the colored complex ***(28)***. Thus, luminescent protein detection systems utilizing chelates complexed to transition metal ions such as europium, or ruthenium should offer greater sensitivity than their colorimetric counterparts without the accompanying hazards associated with radioactivity. The organic component of the complex absorbs light and transfers the energy to the transition metal ion, which subsequently emits light at longer wavelength.

This is demonstrated by substituting europium into the bathophenanthroline–disulfonate complex ***(17)***. This luminescent reagent has been commercialized as SYPRO Rose protein blot stain (Molecular Probes, Eugene, OR). The bathophenanthroline disulfonate-europium complex can detect as little as 8 ng of protein immobilized on nitrocellulose or PVDF membranes. By comparison, the original bathophenanthroline disulfonate–ferrous complex is capable of detecting 600 ng of protein, while the modification employing [^{59}Fe] is capable of detecting 10–25 ng of protein ***(20,21)***. The luminescent stain is readily removed by incubating blots in mildly alkaline solution, is highly resistant to photobleaching and is compatible with popular downstream biochemical characterization procedures including immunoblotting, lectin blotting, and mass spectrometry ***(17)***. Disadvantages of the bathophenanthroline disulfonate–europium stain are that the dye can only be adequately visualized using 302 nm UV-B epi-illumination and the dye exhibits intense 430 nm (blue) fluorescence emission as well as the desired red emission maxima of 595 and 615 nm.

Subsequently, SYPRO Rose Plus protein blot stain, an improved europium-based metal chelate stain roughly 10 times brighter than the original bathophenanthroline disulfonate-europium stain, was introduced ***(25,26)***. The intense blue fluorescence from uncomplexed ligand, observed in the original stain, was eliminated by employing a thermodynamically more stable europium complex. Due to improved absorption properties, the stain could now be readily visualized with UV-A UV-B or UV-C epi-illumination. Just as with the bathophenanthroline disulfonate–europium stain, SYPRO Rose Plus stain is easily removed by increasing solution pH. The stain is fully compatible with biotin–streptavidin and immunoblotting detection technologies that use a wide variety of visualization strategies. Neither of the europium-based stains is compatible with laser-based gel scanners as they lack visible excitation peaks. SYPRO Ruby dye is a proprietary ruthenium-based metal chelate stain developed to address the limitations of the SYPRO Rose and SYPRO Rose Plus dyes. SYPRO Ruby protein blot stain visualizes electroblotted proteins on nitrocellulose and PVDF membranes with a detection sensitivity of 0.25–1 ng of protein/mm^2 in slot-blotting applications. Approximately 2–8 ng of protein can routinely be detected by electroblotting, which side-by-side comparisons demonstrate is as sensitive as colloidal gold stain ***(22)***. While colloidal gold staining requires 2–4 h, SYPRO Ruby dye staining is complete in 15 min. The linear dynamic range of SYPRO Ruby protein blot stain is vastly superior to colloidal gold stain, extending over a 1000-fold range. The dye can be excited using a standard 302 nm UV-B transilluminator or using imaging systems equipped with 450-, 473-, 488-, or even 532- nm lasers. Unlike colloidal gold stain, SYPRO Ruby stain does not interfere with mass spectrometry or immunodetection procedures ***(22)***.

SYPRO Ruby protein gel stain and SYPRO Ruby IEF protein gel stains allow one-step, low background staining of proteins in polyacrylamide or agarose gels without resorting to lengthy destaining steps (*see* **Fig. 1**). The linear dynamic range of these dyes extends over three orders of magnitude, thus surpassing silver and Coomassie Blue stains in performance. An evaluation of 11 protein standards ranging in isoelectric point from 3.5 to 9.3 indicates that SYPRO Ruby IEF gel stain is 3–30 times more sensitive than highly sensitive silver stains ***(24)***. Proteins that stain poorly with silver stain techniques are often readily detected by SYPRO Ruby dye ***(27)***. Similar to colloidal Coomassie Blue stain but unlike silver stain, SYPRO Ruby dye stains are end point stains. Thus, staining times are not critical and staining can be performed over night without gels overdeveloping.

A potential disadvantage to detection of proteins using luminescent compared to colorimetric metal chelate stains is the requirement for ancillary equipment such as a laser gel scanner, UV light box, bandpass filters, and photographic or charge-coupled device (CCD) camera. The tremendous improvement in detection sensitivity and linear dynamic range certainly justifies the investment in equipment. Procedures for the detection of electrophoretically

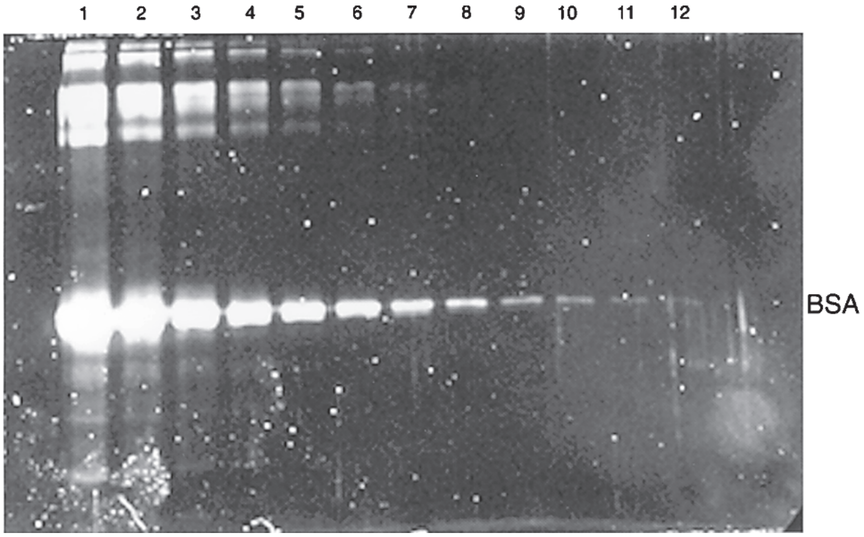

Fig. 1. Protein detection sensitivity in 1-D gels using SYPRO Ruby Protein gel stain: Twofold serial dilutions of solution-quantified bovine serum albumin (P-7656, Sigma Chemical, Saint Louis, MO) electrophoretically separated on 13% Duracryl SDS-polyacrylamide gels (Genomic Solutions, Ann Arbor, MI). After staining with SYPRO Ruby protein gel stain (Molecular Probes, Eugene, OR), gels were imaged using a Lumi-Imager-F1 scanner (Roche Biochemicals, Indianapolis, IN). The 302-nm UV-B transilluminator was used in conjunction with the system's 600-nm emission filter. As little as 250 pg of serum albumin is detectable, with a linearly increasing signal extending to 1000 ng.

separated proteins utilizing colorimetric and luminescent metal chelate stains are presented in this chapter. Methods for the elution of the metal chelate stains are also presented. The procedures are applicable for detection of proteins or peptides in polyacrylamide or agarose gels as well as on nitrocellulose, PVDF, or nylon membranes. Owing to the electrostatic mechanism of the protein visualization methods, metal chelate stains are unsuitable for detection of proteins and peptides immobilized on cationic membranes.

2. Materials

2.1. Colorimetric Detection of Electroblotted Proteins on Membranes

1. Block buffer: 0.1% polyvinylpyrrolidone-40 (PVP-40) in 2% glacial acetic acid.
2. Ferrozine–ferrous stain (stable for at least 6 mo at room temperature): 0.75 m*M* 3-(2-pyridyl)-5,6-*bis* (4-phenylsulfonic acid)-1,2,4-triazine disodium salt

(Ferrozine), 30 m*M* ferric chloride, 15 m*M* thioglycolic acid in 2% glacial acetic acid. Alternatively, commercially prepared stain solutions of Ferrozine–ferrous (Rev–Pro stain kit; Genomic Solutions, Ann Arbor, MI) or Pyrogallol Red-molybdenum (Microprotein-PR kit; Sigma Chemical Company, St. Louis, MO) can be used.

3. 2% Glacial acetic acid.
4. Ferrocyanide–ferric stain (stable for at least 6 mo at room temperature): 100 m*M* sodium acetate, pH 4.0; 100 m*M* potassium ferrocyanide, 60 m*M* ferric chloride.

2.2. Luminescent Detection of Electroblotted Proteins Using Bathophenanthroline Disulfonate-europium (SYPRO Rose Stain)

1. Formate buffer: 100 m*M* formic acid, pH 3.7, 100 m*M* sodium chloride.
2. Bathophenanthroline–europium blot stain (stable for at least 6 mo at room temperature): 1.5 m*M* bathophenanthroline disulfonic acid disodium salt, 0.5 m*M* europium chloride, and 0.2 m*M* EDTA (added from 1000× stock, pH 7.0).

2.3. Luminescent Detection of Electroblotted Proteins Using SYPRO Rose Plus Protein Blot Stain

1. SYPRO Rose Plus protein blot stain kit (Molecular Probes, Inc. cat. #S-12011) The kit contains the following components:
   SYPRO Rose Plus blot wash solution (component A), 200 mL
   SYPRO Rose Plus blot stain solution (component B), 200 mL
   SYPRO Rose Plus blot destain solution (component C), 200 mL

The kit contains sufficient material for staining 10–40 minigel electroblots or four large-format electroblots (20 × 20 cm). The SYPRO Rose Plus solutions may be reused up to four times with little loss in sensitivity.

2.4. Luminescent Detection of Electroblotted Proteins Using SYPRO Ruby Protein Blot Stain

1. SYPRO Ruby protein blot stain (Molecular Probes, Inc.) is provided in a unit size of 200 mL. The 200-mL volume is sufficient for staining 10–40 minigel electroblots or four large-format electroblots (20 × 20 cm). SYPRO Ruby protein blot stain may be reused up to four times with little loss in sensitivity.
2. 7% Acetic Acid, 10% methanol.

2.5. Luminescent Detection of Proteins in SDS-Polyacrylamide Gels Using SYPRO Ruby Protein Gel Stain

1. SYPRO Ruby protein gel stain (Molecular Probes, Inc.) is provided ready to use, in either 200 mL volume (will stain ~four minigels) or 1 L volume (~ 20 minigels or 2–3 large-format gels).
2. 7% Acetic Acid, 10% methanol.

2.6. Luminescent Detection of Proteins in Isoelectric Focusing (IEF) Gels Using SYPRO Ruby IEF Protein Gel Stain

1. SYPRO Ruby IEF protein gel stain (Molecular Probes, Inc.) is supplied as a 400-mL ready-to-use staining solution, sufficient to stain ~10 IEF minigels or two standard flatbed IEF gels. Use caution when handling the SYPRO Ruby IEF protein gel stain as it contains a strong acid that can cause burns.

2.7. The Elution of Metal Chelate Stains

1. 50 m*M* Tris-HCl, pH 8.8, 200 m*M* NaCl, 20 m*M* EDTA (for the Ferrozine–ferrous stain).
2. 200 m*M* Sodium carbonate, 100 m*M* EDTA, pH 9.6 (for the Ferrozine–ferrous stain enhanced with the ferrocyanide–ferric stain and for the bathophenanthroline–europium blot stain).
3. 200 m*M* Sodium carbonate; 100 m*M* EDTA, pH 9.6 in 30% methanol (for the bathophenanthroline-europium gel stain).
4. SYPRO Rose Plus blot destain solution (component C) (for the SYPRO Rose Plus protein blot stain.)

3. Methods

3.1. Colorimetric Detection of Electroblotted Proteins on Membranes

The colorimetric metal chelate stains allow rapid visualization of proteins on solid-phase supports with detection sensitivities that are comparable to Coomassie Brilliant Blue staining ***(13–16)***. Detection sensitivity of the Ferrozine–ferrous stain can be enhanced to a level comparable to silver staining by further incubating membranes in ferrocyanide-ferric stain ***(13,15)***. The colorimetric metal chelate stains are fully reversible and compatible with Edman-based protein sequencing, lectin blotting, mass spectrometry and immunoblotting ***(13–16)***.

1. After electroblotting, vacuum slot blotting, or dot blotting, membranes are completely immersed in block buffer for 10 min. Blocking and staining steps are performed on a rotary shaker (50 rpm).
2. Thoroughly immerse membranes in Ferrozine-ferrous stain for 10–15 min until purple bands or spots appear.
3. Unbound dye is removed by several brief rinses in 2% glacial acetic acid until the membrane background appears white. Shaking can be performed manually using wash volumes roughly 2× greater than in the blocking and staining steps.
4. If increased sensitivity is desired, the blot can be double stained by subsequently incubating in the ferrocyanide-ferric stain for 10–15 min (*see* **Notes 1–3**).
5. Unbound dye is removed by several brief washes in 100 m*M* sodium acetate, pH 4.0 (*see* **Note 4**). Shaking can be performed manually using wash volumes roughly 2× greater than in the blocking and staining steps.
6. Stained proteins are visualized by eye and quantified using a CCD camera (*see* **Note 5**).

3.2. Luminescent Detection of Electroblotted Proteins Using Bathophenanthroline Disulfonate-Europium (SYPRO Rose Stain)

Bathophenanthroline disulfonate–europium complex (SYPRO Rose protein blot stain) is a medium sensitivity metal chelate stain that offers the same advantages as colorimetric stains, but with the additional benefits of a 500-fold linear dynamic range and detection sensitivity of <15–30 ng of protein ***(17)***. The stain can be visualized using 302 nm UV epi-illumination and emits at 590 and 615 nm. There is also a blue 450 nm emission, however, due to uncomplexed ligand. Bathophenanthroline disulfonate–europium complex is readily removed by incubation of blots in mildly alkaline solution.

1. Following electroblotting, vacuum slot blotting, or dot blotting, membranes are washed 2× for 30 min with formate buffer followed by 4× for 30 min with deionized water (*see* **Note 6**). All washing and staining steps are performed on a rotary shaker (50 rpm).
2. Membranes are completely immersed in the bathophenanthroline-europium blot stain for 15 min.
3. Unbound dye is removed by washing 4–6× for 1 min in deionized water (*see* **Note 7**).
4. Membranes are dried at room temperature or in a 37°C drying oven (for quicker results) following the washing steps (*see* **Note 8**).
5. Stained proteins are visualized by reflective UV illumination (*see* **Note 9**).

3.3. Luminescent Detection of Electroblotted Proteins Using SYPRO Rose Plus Protein Blot Stain

SYPRO Rose Plus protein blot stain is an improved europium-based complex with a narrow emission maximum centered at 615 nm. The blue fluorescence observed with SYPRO Rose dye has been eliminated with the new stain and SYPRO Rose Plus dye is readily excited using UV-A, UV-B, or UV-C epi-illumination. Transillumination is not recommended. Typically 2–8 ng of electroblotted protein may be detected using the stain. SYPRO Rose Plus protein blot stain is easily removed by incubation in a mildly alkaline solution. The stain can not be visualized using a laser-based gel scanner, but is fully compatible with mass spectrometry and immunoblotting technologies.

1. Following electroblotting, vacuum slot blotting, or dot blotting, membranes are completely immersed in SYPRO Rose Plus protein blot wash solution and incubated at room temperature for 15 min in a small, polypropylene staining dish. Perform all washing and staining steps with continuous, gentle agitation (ideally, on an orbital shaker at 50 rpm).
2. Repeat the wash step.
3. Incubate the membrane in four changes of deionized water for 10 min each.

4. Completely immerse the membrane in SYPRO Rose Plus protein blot stain reagent for 15 min.
5. Wash the membrane 4–6× for 1 min in deionized water, to remove excess dye from the membrane. Stained membranes should be monitored using UV epi-illumination periodically to determine whether background luminescence has been washed away (*see* **Note 10**).
6. Blots treated with the SYPRO Rose Plus blot stain are best preserved by allowing membranes to air dry. After staining, wet membranes should not be touched, since residue found on latex gloves may destroy the staining pattern. Use forceps to handle wet blots. Once dry, membranes can be handled freely.
7. Stained proteins are visualized by reflective UV illumination (*see* **Note 11**).

3.4. Luminescent Detection of Electroblotted Proteins Using SYPRO Ruby Protein Blot Stain

SYPRO Ruby protein blot stain is suitable for visualizing proteins on nitrocellulose or PVDF membranes using UV transillumination, UV epi-illumination, or laser excitation at 450–532 nm ***(22)***. Roughly 2–8 ng of protein can routinely be detected on electroblots and the linear dynamic range of detection is roughly 1000-fold. Unlike other sensitive detection technologies such as colloidal gold stain, SYPRO Ruby protein blot stain is fully compatible with mass spectrometry and Edman-based sequencing. The stain is fairly permanent, but is lost during protein blocking steps associated with Western blotting. Thus, the fluorescent pattern must be recorded and documented prior to immunodetection of a specific target on the blot.

3.4.1. Staining Proteins After Electroblotting to Nitrocellulose Membranes

1. Following electroblotting, vacuum slot blotting, or dot blotting to nitrocellulose, membranes are completely immersed in 7% acetic acid, 10% methanol and incubated at room temperature for 15 min in a small, polypropylene staining dish. Perform all washing and staining steps with continuous, gentle agitation (ideally, on an orbital shaker at 50 rpm).
2. Incubate the membrane in four changes of deionized water for 5 min each.
3. Completely immerse the membrane in SYPRO Ruby protein blot stain reagent for 15 min.
4. Wash the membrane 4–6× for 1 min in deionized water. This wash serves to remove excess dye from the membrane. Membranes stained with SYPRO Ruby protein blot stain should be monitored using UV epi-illumination periodically to determine if background luminescence has been washed away (*see* **Notes 12** and **13**).
5. Blots treated with the SYPRO Ruby protein blot stain can be preserved by allowing membranes to air-dry. After staining, wet membranes should not be touched, since residue found on latex gloves may destroy the staining pattern. Use forceps to handle wet blots. Once dry, membranes can be handled freely.

6. Stained proteins may be visualized by reflective or transmissive UV illumination or using a laser-based gel scanner (*see* **Notes 12** and **13**).

3.4.2. Staining Proteins After Electroblotting to PVDF Membranes

1. Following electroblotting, vacuum slot blotting, or dot blotting, to a sheet of PVDF membrane, allow the membrane to dry completely.
2. Float the membrane face down in 7% acetic acid, 10% methanol and incubate for 15 min. Perform all washing and staining steps with continuous, gentle agitation (e.g., on an orbital shaker at 50 rpm).
3. Incubate the membrane for 5 min each in four changes of deionized water.
4. Float the membrane face down in SYPRO Ruby protein blot stain reagent for 15 min.
5. Wash the membrane 2–3× for 1 min in deionized water. This wash serves to remove excess dye from the membrane. Membranes stained with SYPRO Ruby protein blot stain should be monitored using UV epi-illumination periodically to determine if background luminescence has been washed away (*see* **Notes 12** and **13**).
6. Blots treated with the SYPRO Ruby protein blot stain are best preserved by allowing the membranes to air-dry. After staining, wet membranes should not be touched, since residue found on latex gloves may destroy the staining pattern. Use forceps to handle wet blots. Once dry, the membranes can be handled freely.
7. Stained proteins may be visualized by reflective or transmissive UV illumination or using a laser-based gel scanner (*see* **Notes 12** and **13**).

3.5. Luminescent Detection of Proteins in Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gels Using SYPRO Ruby Protein Gel Stain

SYPRO Ruby protein gel stain is an ultrasensitive dye for detecting proteins separated by SDS-polyacrylamide or 2-D polyacrylamide gel electrophoresis (PAGE). The background fluorescence is low, the stain's linear dynamic range extends over three orders of magnitude, and shows low protein-to-protein variation. The stain is more sensitive than colloidal Coomassie Blue stain and equal in sensitivity to the best silver stains available. The stain is ready-to-use, and gels cannot over stain. Optimal staining requires about 3–4 h. Staining times are not critical, however, and staining can be performed overnight. SYPRO Ruby protein gel stain will not stain extraneous nucleic acids, and it is compatible with further downstream microchemical processing. The stain does not interfere with subsequent analysis of proteins by Edman-based sequencing or mass spectrometry. SYPRO Ruby protein gel stain can be used with many types of gels, including 2-D gels, Tris-glycine SDS gels, Tris-tricine precast SDS gels, and nondenaturing gels. SYPRO Ruby protein gel stain is also compatible with gels adhering to plastic backings, although the signal from the inherent blue fluorescence of the plastic must be removed with an appropriate emission filter. The stain is suitable for peptide mass profiling using MALDI-TOF mass spectrometry ***(25–27).*** Stained gels can be visualized with a 302-nm UV transilluminator, various laser scanners, or other blue light-emitting sources. The dye maximally emits at about 610 nm.

1. Prepare and run SDS-polyacrylamide or 2-D polyacrylamide gels according to standard protocols. Perform staining with SYPRO Ruby protein gel stain using continuous, gentle agitation (e.g., on an orbital shaker at 50 rpm). For small gels, use circular staining dishes on orbital shakers if possible.
2. Clean and *thoroughly rinse* the staining dishes before use (*see* **Note 14**).
3. Fix the gel in 10% methanol and 7% acetic acid in a plastic dish for 30 min. This step improves the sensitivity of the stain in 2-D gels, but is optional for 1-D SDS-PAGE gels.
4. Pour the staining solution into a small, clean plastic dish. For one or two standard-size minigels, use ~50–100 mL of staining solution; for larger gels, use 500–750 mL (*see* **Note 15**).
5. Place the gel into fresh staining solution (*see* **Note 16**). Protect the gel and staining solution from light at all times by covering the container with a lid or with aluminum foil.
6. Gently agitate the gel in stain solution at room temperature. The staining times range from 90 min to 3 h, depending on the thickness and percentage of polyacrylamide in the gel. Specific staining can be seen in as little as 30 min. However, a minimum of 3 h of staining is required for the maximum sensitivity and linearity. For convenience, gels may be left in the dye solution overnight or longer without over staining.
7. After staining, rinse the gel in deionized water for 30–60 min to decrease background fluorescence. To further decrease background fluorescence the gel can be washed in a mixture of 10% methanol and 7% acetic acid for 30 min instead of water (*see* **Note 17**). This is especially recommended for 2-D gels. The gel may be monitored periodically using UV illumination to determine the level of background fluorescence (*see* **Note 18**).
8. To dry the stained gel for permanent storage, incubate the gel in a solution of 2% glycerol for 30 min. Dry the stained gel using a gel dryer by standard methods. Note that proteins present at very low levels may no longer be detectable after gel drying.
9. Stained proteins in wet or dried gels may be visualized by reflective or transmissive UV illumination or using a laser-based gel scanner (*see* **Note 18**).

3.6. Luminescent Detection of Proteins in Gels Using SYPRO Ruby IEF Protein Gel Stain

SYPRO Ruby IEF protein gel stain is a ready-to-use, ultrasensitive, luminescent protein stain created especially for the analysis of proteins in IEF gels. This fluorescent stain attains comparable sensitivity to that of the best silver-staining techniques. Staining protocols are simple, the stain is ready-to-use, and it cannot overstain. It will not stain extraneous nucleic acids and the stain detects glycoproteins and other difficult-to-stain proteins. It does not interfere with subsequent analysis of proteins by Edman-based sequencing or mass spectrometry. SYPRO Ruby IEF protein gel stain is also compatible with agrose gels or polyacrylamide gels adhering to plastic backings.

1. Prepare and run IEF gels according to standard protocols. The staining technique is appropriate for carrier ampholyte isoelectric focusing or immobilized pH gradient (IPG) gels ***(24)***. Perform SYPRO Ruby IEF gel staining with continuous, gentle agitation (e.g., on an orbital shaker at 50 rpm). For small gels, use circular staining dishes on orbital shakers if possible.
2. Clean and *thoroughly rinse* the staining dishes before use. (*see* **Note 20**).
3. Incubate the gel in the undiluted stain overnight. (*see* **Note 19**). **Caution: SYPRO Ruby IEF protein gel stain contains a caustic acid. The stain should be handled with care, using protective clothing, eye protection, and gloves.**
4. Rinse the gel in four changes of deionized water over 2 h to decrease background fluorescence. The gel may be monitored periodically using UV transillumination (*see* **Note 21**) to determine the level of background fluorescence. For IEF gels with plastic backings, the gel separates from the backing during this water wash. It is important that the backing be removed from the gels as it has high inherent fluorescence.
5. To dry the stained gel for permanent storage, incubate the gel in a solution of 2% glycerol for 30 min. Dry the stained gel using a gel dryer by standard methods. Note that proteins present at very low levels may no longer be detectable after gel drying.
6. Stained proteins in wet or dried gels may be visualized by reflective or transmissive UV illumination or using a laser-based gel scanner (*see* **Note 21**).

3.7. Elution of Metal Chelate Stains

The Ferrozine-ferrous, Pyrogallol Red–molybdate, Ferrocyanide–ferric, colorimetric metal chelate stains as well as the luminescent bathophenanthroline disulfonate–europium and SYPRO Rose Plus protein blot stains are readily removed from electroblotted proteins by incubation in mildly alkaline solution. SYPRO Ruby protein blot stain is not readily reversible by similar methods, although it is slowly removed from proteins during blocking and incubation steps commonly used in immunoblotting procedures. The gel stains are also not readily destained.

The Ferrozine–ferrous stain can be eluted by immersing the blots in 50 m*M* Tris-HCl, pH 8.8, 200 m*M* NaCl, 20 m*M* EDTA for 15 min on a rotary shaker (50 rpm). Blots treated with the Ferrozine–ferrous stain followed by enhancement with the ferrocyanide–ferric stain require harsher elution conditions. This stain is eluted by incubation in 200 m*M* sodium carbonate, 100 m*M* EDTA, pH 9.6 for 10 min. As the bathophenanthroline-europium and SYPRO Rose Plus stains can be observed only upon UV illumination, they do not require elution if subsequent colorimetric detection procedures are to be performed ***(29)***. If concerned about interference with subsequent procedures, however, the stains can be eluted from blots by incubation in 200 m*M* sodium carbonate, 100 m*M* EDTA, pH 9.6 or using SYPRO Rose Plus destain solution (component C). All the metal chelate stains mentioned above may be destained using the following protocol:

1. Incubate the membrane in appropriate destain reagent for 15 min with continuous gentle agitation.
2. Rinse twice for 1 min each in deionized water.

4. Notes

4.1. Colorimetric Detection of Proteins on Nitrocellulose, PVDF, or Nylon Membranes

1. Although enhancement of the Ferrozine–ferrous stain with ferrocyanide–ferric stain substantially increases detection sensitivity, the double stain is also more difficult to elute than the Ferrozine–ferrous stain alone (*see* **Subheading 3.7**, for methods of stain reversal).
2. The ferrocyanide-ferric stain may form a precipitate after long–term storage. The precipitate is easily resuspended by vigorous shaking or sonication.
3. If a dried nitrocellulose membrane is incubated in the ferrocyanide–ferric stain, a patchy background may result that is difficult to destain. Dry blots should be rehydrated briefly in deionized water prior to incubation in the ferrocyanide–ferric stain.
4. The number of washes necessary to remove background staining may vary slightly. The ferrocyanide–ferric stain may initially remain bound to the nitrocellulose membrane but the membrane background will become white with sufficient washing.
5. Membranes may be imaged transmissively or reflectively. Transmissive imaging is preferred over conventional reflective imaging as it improves signal detection while maintaining a white background. Furthermore, in some cases the protein sample penetrates through the membrane support to the reverse side (particularly with vacuum slot blotters). The metal chelate complexes stain proteins present throughout the thickness of the membrane and on both surfaces but reflective scanning only detects signal on the front surface, leading to a poorer linear dynamic range of quantitation. This problem is alleviated with transmissive scanning. Typically, a 45 W white light box is used in conjuction with a 450 ± 70 nm bandpass filter to enhance image contrast when imaging Ferrozine–ferrous or Pyrogallol Red-molybdenum stains. For blue stains such as Ferrozine-ferrous followed by the ferrocyanide-ferric stain, a 600 ± 70 nm bandpass filter may enhance the image contrast. Because the membrane support blocks a

substantial amount of transmitted light, care must be taken to completely mask the sample with black cardboard for transmissive imaging.

4.2. Luminescent Detection of Electroblotted Proteins Using Bathophenanthroline Disulfonate-Europium (SYPRO Rose Stain)

6. Since formate ions may chelate europium ions, the washes with deionized water are crucial for complete removal of the formate buffer. Otherwise, the staining solution may be inactivated. Care should be taken to aspirate off all the solution between washes.
7. Removal of unbound bathophenathroline-europium stain can not be visually monitored as with the colorimetric stains. We have found that washing 4× for 1 min is effective. However, the number and duration of washes may vary from case to case (depending on the size of the membrane and the volume of each wash). Therefore, optimal washing should be determined empirically for each application.
8. After staining, wet membranes should not be touched since residue found on latex laboratory gloves may destroy the stain. Once dry, membranes can be handled freely. Since water is known to quench europium luminescence, drying the membrane also serves to enhance the signal (about twofold).
9. Images are best obtained using a cooled CCD-camera by digitizing at about 1024 × 1024 picture elements (pixels) resolution with 12- or 16-bit gray scale levels assigned per pixel. To visualize the stain, the front face of membranes should be illuminated with the UV source: a hand-held, UV-B (302 nm) light, a UV light box placed on its side, or a top illuminating system such as the Bio-Rad Fluor-S imager. Direct transillumination through the blotting membrane yields unsatisfactory results. SYPRO Rose protein blot stain is best visualized using a 490-nm longpass filter such as a SYPRO protein gel photographic filter (Molecular Probes, Inc., cat. no. S-6656) or a 600-nm bandpass filter.

4.3. Luminescent Detection of Electroblotted Proteins Using SYPRO Rose Plus Protein Blot Stain

10. Removal of unbound bathophenathroline–europium stain cannot be visually monitored as with the colorimetric stains. We have found that washing 4× for 1 min is effective. However, the number and duration of washes may vary from case to case (depending on the size of the membrane and the volume of each wash). Therefore, optimal washing should be determined empirically for each application.
11. Proteins stained with SYPRO Rose Plus protein blot stain are readily visualized by eye using epi-illumination with a UV light source. Illuminate the

front face of the membranes using a hand-held, UV-B (302 nm) light source, a UV light box placed on its side, or a top illuminating system such as the Bio-Rad Fluor-S™ imager. For greatest sensitivity and accurate quantitation, photograph the blot using Polaroid photography with a 490-nm longpass filter such as the SYPRO protein gel photographic filter (Molecular Probes, Inc., cat. no. S-6656) or use a computerized CCD camera-based image analysis system equipped with a 600-nm bandpass filter. Direct transillumination through the blotting membrane yields unsatisfactory results.

4.4. Luminescent Detection of Electroblotted Proteins Using SYPRO Ruby Protein Blot Stain

12. Proteins stained with SYPRO Ruby protein blot stain are readily visualized by eye using epi-illumination with UV light source. Illuminate the front face of the membranes using a hand-held, UV-B (302 nm) light source, a UV light box placed on its side, or a top illuminating system. Alternatively, use direct transillumination through the blotting membrane. For greater sensitivity and accurate quantitation, photograph the blot using Polaroid® photography or a computerized CCD camera-based image analysis system equipped with a 490-nm longpass filter such as the SYPRO protein gel photographic filter (Molecular Probes, Inc., cat no. S-6656) or a 600-nm bandpass filter. Laser-based imaging systems can also be used.
13. Images are best obtained using a cooled CCD-camera by digitizing at about 1024 × 1024 picture elements (pixels) resolution with 12- or 16-bit gray scale levels assigned per pixel. To visualize the stain, the front face of membranes should be illuminated with the UV source: a hand-held, UV-B (302 nm) light, a UV light box placed on its side, or a top illuminating system such as the Bio-Rad Fluor-S imager. Satisfactory results are obtained from direct transillumination through the blotting membrane as well. SYPRO Ruby protein blot stain is best visualized using a 490-nm longpass filter such as a SYPRO protein gel photographic filter (Molecular Probes, Inc., cat. no. S-6656) or a 600 nm bandpass filter. Proteins stained with the dye can also be visualized using imaging systems equipped with 450-, 473-, 488-, or even 532-nm lasers.

4.5. Luminescent Detection of Proteins in SDS-Polyacrylamide Gels Using SYPRO Ruby Protein Gel Stain

14. Polypropylene dishes, such a Rubbermaid® Servin' Savers, are the optimal containers for staining because the high-density plastic adsorbs only a minimal amount of the dye. Clean and rinse the staining containers well before use, as detergent will interfere with staining. It is best to rinse the containers with ethanol before use. For small gels, circular staining dishes provide the

best fluid dynamics on orbital shakers, resulting in less dye aggregation and better staining. For large format 2-D gels, polyvinyl chloride photographic staining trays, such as Photoquip Cesco-Lite 8 in. × 10 in. photographic trays also work well. Glass dishes are not recommended.

15. Minimal staining volumes for typical gel sizes are as follows:
    50 mL, for 8 cm × 10 cm × 0.75 mm gels (minigels)
    330 mL, for 16 cm × 20 cm × 1 mm gels
    500 mL, for 20 cm × 20 cm × 1 mm gels
    or ~ 10 times the volume of the gel for other gel sizes
16. Use only fresh staining solution for optimal sensitivity. Longer staining times result in greater sensitivity. Using too little stain will lower the sensitivity.
17. Always store gels in the dark to prevent photobleaching. When gels are stored in the staining solution, the signal decreases somewhat after several days; however, depending on the amount of protein in bands of interest, gels may retain a usable signal for many weeks.
18. The stained gel is best viewed on a standard 302 nm UV or a blue-light transilluminator. Gels may also be visualized using various laser scanners: 473 nm (SHG) laser, 488 nm argon-ion laser, 532 nm (YAG) laser. Alternatively, use a xeon arc lamp, blue fluorescent light, or blue light-emitting diode (LED) source. Gels may be photographed by Polaroid or CCD camera. Use Polaroid 667 black-and-white print film and the SYPRO protein gel stain photographic filter (Molecular Probes, Inc., cat. no. S-6656). Exposure times vary with the intensity of the illumination source; for an f-stop of 4.5, roughly 1–3 s should be required.

4.6. Luminescent Detection of Proteins in IEF Gels Using SYPRO Ruby IEF Protein Gel Stain

19. Minimal staining volumes for typical gel sizes are as follows:
    50 mL, for 6 cm × 9 cm × 1 mm gels (minigels)
    150 mL, for 22 cm × 22 cm × 1 mm (Multiphor II format gels, Amersham-Pharmacia Biotech)
    Or approx 10 times the volume of the gel for other gel sizes.
20. Polypropylene dishes, such as Rubbermaid® Servin' Savers, are the optimal containers for staining because the high-density plastic adsorbs only a minimal amount of the dye. Clean and rinse the staining containers well before use as detergent will interfere with staining. It is best to rinse the containers with ethanol before use. For small gels, circular staining dishes provide the best fluid dynamics on orbital shakers, resulting in less dye aggregation and better staining. For large-format 2-D gels, polyvinyl chloride photographic staining trays, such as Photoquip Cesco-Lite 8 in. × 10 in. photographic trays also work well. Glass dishes are not recommended.

21. The stained gel is best viewed on a standard 302-nm UV or a blue-light transilluminator. Gels may also be visualized using various laser scanners: 473-nm (SHG) laser, 488 nm argon-ion laser, 532-nm (YAG) laser. Alternatively, use a xenon arc lamp, blue fluorescent light, or blue light-emitting diode (LED) source. Gels may be documented by conventional photography using the SYPRO protein gel stain photographic filter (Molecular Probes, Inc., cat. no. S-6656) or using a CCD camera equipped with 600-nm bandpass filter. Exposure times vary with the intensity of the illumination source; for an f-stop of 4.5, roughly 1–3 s should be required.

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Detection of Proteins in Polyacrylamide Gels by Fluorescent Staining

Michael J. Dunn

1. Introduction

Techniques of polyacrylamide gel electrophoresis are often the method of choice for the analysis of patterns of protein expression in a wide variety of complex systems. In particular, two-dimensional polyacrylamide gel electrophoresis (2-DE) is the core technology for separating complex protein mixtures in the majority of proteome projects ***(1)***. This is due to its unrivalled power to separate simultaneously thousands of proteins and to indicate post-translational modifications that result in alteration in protein pI or M_r. Additional advantages are the high sensitivity with which the separated proteins can be visualised, the availability of sophisticated computer image analysis software for the qualitative and quantitative analysis of differential patterns of protein expression ***(2)***, and the relative ease with which proteins from 2-DE gels can be identified by mass spectrometry (MS) ***(3, 4)***.

The successful use of polyacrylamide gel electrophoresis as an analytical tool for investigating protein expression and as a micro-preparative procedure for protein identification and characterisation depends on the availability of detection methods that are both quantitative and sensitive. Coomassie Brilliant Blue R-250 (CBB R-250) has been used for many years as a general protein stain following gel electrophoresis. Its restricted dynamic range limits its suitability for accurate quantitative analysis, although it is compatible with most methods of chemical characterisation. However, CBB R-250 has a limited sensitivity of around 200–500 ng protein per spot ***(5)***. The development of colloidal CBB staining using CBB G-250 ***(6)*** provided a significant increase in sensitivity at around 10 ng protein. However, the trend towards the use of thinner gels and the need to detect small amount of protein within single bands or spots resolved by

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one- or two-dimensional electrophoresis have necessitated the development of more sensitive detection methods ***(5).***

Silver staining for the detection of proteins following gel electrophoresis was first reported in 1979 by Switzer et al ***(7),*** resulting in a major increase in the sensitivity of protein detection. More than 100 publications have subsequently appeared describing variations in silver staining methodology **(5, 8).** This group of procedures is generally accepted to be around 100 times more sensitive than methods using CBB R-250, being able to detect low ng amounts of protein per gel band or spot. Although silver staining has become the most widely used technique for high sensitivity visualisation of gel separated proteins, its variable binding characteristics towards many proteins and its relatively restricted dynamic range limit the accuracy and reliability of quantitation. Moreover, saturated protein bands and spots tend to be negatively stained making their quantitation impossible. Standard methods of silver staining are also often not compatible with chemical characterisation by mass spectrometry as the glutaraldehyde and formaldehyde present results in alkylation of α- and ε-amino groups of proteins. To overcome this problem, silver staining protocols compatible with mass spectrometry in which glutaraldehyde is omitted have been developed ***(9, 10)*** but these suffer from a decrease in sensitivity of staining and a tendency to a higher background.

Many of the problems associated with silver staining can be overcome using detection methods based on the use of fluorescent compounds ***(5).*** This group of methods are highly sensitive and generally exhibit excellent linearity and a high dynamic range, making it possible to achieve good quantitative analysis particularly if a suitable imaging device is used. In addition, these methods should in general have good compatibility with protein identification by MS. Two approaches can be used, the first being to couple the proteins with a fluorescently labelled compound prior to electrophoresis. Examples of such compounds are dansyl chloride ***(11),*** fluorescamine (4-phenyl-[furan-2(3H),1-phthalan]-3,3 -dione) ***(12),*** o-pththaldialdehyde (OPA) (13), and MDPF (2-methoxy-2, 4-diphenyl-3(2H)-furanone) ***(14).*** The latter reagent has a reported sensitivity of 1 ng protein/band and is linear over the range 1–500 ng protein/band.

The main disadvantage of pre-electrophoretic staining procedures is that they can cause protein charge modifications, for example fluorescamine converts an amino group to a carboxyl group when it reacts with proteins. Such modifications usually do not compromise SDS-PAGE unless a large number of additional charged groups are introduced into the protein. However, they result in altered mobility during other forms of electrophoresis, resulting in altered separations by native PAGE, IEF and 2-DE. More recently, compounds that react with cysteine or lysine residues have been described and used successfully for 2-DE separations. The cysteine-reactive reagent monobromobimane ***(15)*** has been used to label proteins prior to analysis by 2-DE ***(16).*** Using a cooled CCD camera to measure fluorescence, the limit of detection was found to be 1 pg protein/spot ***(17).***

In an alternative approach, a pre-electrophoretic fluorescent staining method based on the labelling of protein samples with *N*-hydroxy succinimidyl ester derivatives of fluorescent cyanine (Cy) dyes and known as two-dimensional difference gel electrophoresis (2-D DIGE) is currently being widely used ***(18, 19).*** This approach has the advantage that a pair of protein samples can be labelled separately with Cy3 and Cy5 derivatives. The two samples can be mixed and then separated together on the same 2-DE gel. The resulting 2-DE gel is then scanned to acquire the Cy3 and Cy5 images separately. Improved quantitative accuracy of comparison of multiple pairs of samples can be achieved using a pooled internal standard labelled with a third dye, Cy2 ***(20, 21).*** Recently saturation labeling with cysteine-reactive cyanine fluorescent dyes has been described ***(22).*** This technique provides increased sensitivity for expression profiling of scarce samples such as laser-microdissected clinical specimens ***(23).***

Another approach to overcoming the problems associated with charge modification during the IEF dimension is to label the proteins while present in the first dimension gel after IEF, prior to the second dimension separation by SDS-PAGE. Two fluorescent labels that have been used in this way are MDPF ***(24)*** and a fluorescent maleimide derivative ***(25).***

An alternative approach, which also overcomes the problem of protein charge modifications, is to label the proteins with fluorescent molecules such as 1-aniline-8-naphthalene sulphonate (ANS) ***(26)*** and OPA after the electrophoretic separation has been completed. However, these two methods suffer the disadvantage of relative insensitivity. Additional post-electrophoretic fluorescent staining reagents, such as SYPRO Orange and Red, have been described ***(5, 27, 28).*** These stains have a high sensitivity (4–10 ng protein/band) and excellent linearity with a high dynamic range. Using a fluorescent imaging device, the SYPRO dyes have been shown to be linear over three orders of magnitude in protein quantity ***(28).*** The other advantage of this method is that staining can be achieved in only 30 min, compared with staining with silver and CBB R-250 which can take from 2 hr to overnight. Gels can be stained without fixation so that they can be subjected to subsequent Western blotting procedures. However, staining with these reagents requires that the proteins are complexed with SDS, so that if the gels are fixed prior to staining or electrophoresis is carried out in the absence of this detergent, then the gels must be incubated in a solution of SDS prior to staining. Moreover, these dyes require 7% acetic acid and organic solvent in the staining solution, which can cause problems in electroblotting, electroeleution and measurement of enzyme activity. The dye SYPRO Tangerine was subsequently developed to overcome these shortcomings ***(29).***

Recently, epiconnone, a non-fluorescent azophilone that fluoresces when it binds to proteins, has been found to a useful fluorescent stain for gel separated proteins ***(30).*** It is marketed under the trade names Deep Purple (GE Healthcare) and Lava Purple (Fluorotechnics) and has been found to be very sensitive and linear over four orders of magnitude ***(31).***

SYPRO Ruby is a ruthenium-based metal chelate stain that binds to the basic amino acid residues in proteins ***(32, 33).*** The advantage of this dye over the other SYPRO stains is that the presence of SDS is not required for binding so that gels can be fixed in the normal way and then simply soaked in the SYPRO Ruby staining solution for a minimum of 3 hr. In addition, SYPRO Ruby has a sensitivity similar to the most sensitive silver stains ***(32, 34),*** and it has been reported that some proteins that stain poorly with silver techniques are readily detected by SYPRO Ruby ***(33).*** In addition, staining times are not critical and staining can be performed overnight without gels overdeveloping. Together with its very high dynamic range of over 1000-fold, these properties make SYPRO Ruby an excellent choice for quantitative applications. It also has been shown to be linear over three orders of magnitude and to have excellent compatibility with chemical characterisation methods ***(35, 36).*** The original formulation of SYPRO Ruby required specific fixation in 7% acetic acid and 10% methanol, while a more recent formulation of the stain is tolerant to a wide range of fixatives ***(37).*** An additional advantage of SYPRO Ruby is that it can be used for multiplexed staining in combination with florescence detection of glycoproteins with Pro-Q Emerald ***(38)*** and phosphoproteins with Pro-Q Diamond ***(39).*** A 2-DE gel separation of rat heart proteins visualised with SYPRO Ruby is shown in **Fig. 1.**

2. Materials

1. All solutions should be freshly prepared, and overnight storage is not recommended.
2. Fixation solution: 10% (v/v) methanol, 7% (v/v) acetic acid (*see* **Note 1**).
3. SYPRO Ruby staining solution (*see* **Note 2**).
4. Washing solution: 10% (v/v) methanol, 7% (v/v) acetic acid (*see* **Note 3**).

3. Method

Note: All incubations are carried out at room temperature with gentle agitation.

1. After electrophoresis, fix the gel immediately (*see* **Note 4**) in 500 mL (*see* **Note 5**) of fixation solution for a minimum of 1 h at room temperature (*see* **Note 6**).
2. Place the gel into 500 mL of SYPRO Ruby protein gel stain solution for a minimum of 3 h (*see* **Note 7**).
3. Incubate the gel in 500 mL of washing solution for 30 to 50 min to wash excess dye out of the polyacrylamide matrix (*see* **Note 8**).
4. Image the gel (*see* **Note 9**).

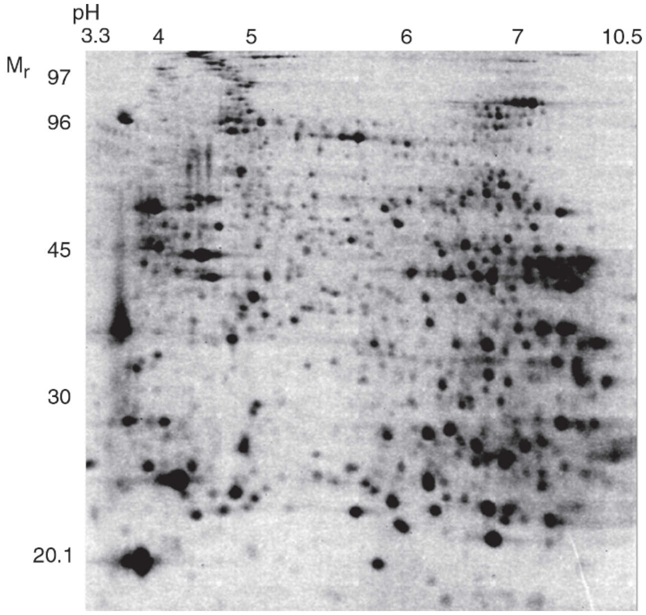

Fig. 1. A 2-DE separation of rat heart proteins visualised by SYPRO Ruby staining. Note that the fluorescent image is shown as an inverted image to show black spots on a white background for convenient viewing. The first dimension was pH 3–10 NL IPG IEF and the second dimension was 12% T SDS-PAGE. The scale at the top indicates the non-linear pH gradient used in the first IPG 3–10 NL IEF dimension, while the scale at the left indicates $M_r \times 10^{-3}$.

4. Notes

1. Methanol may be substituted with ethanol without loss of sensitivity, and a range of other fixatives can be used ***(36).***
2. SYPRO Ruby protein gel stain is only available as a pre-made solution from the manufacturers (Invitrogen, Molecular Probes, Paisley, UK).
3. Methanol may be replaced with ethanol in the washing solution.
4. Gloves should be worn at all stages when handling gels, particularly when they will be used for subsequent chemical characterisation, to prevent contamination with keratin proteins from the skin.
5. Volumes of the solutions used at all stages should be sufficient such that the gel is totally immersed. If the volume of solution is insufficient for total immersion, staining will be uneven and the gel surface can dry out. The volumes given here are suitable for a 24 × 20 cm 2-D gel.
6. High-percentage polyacrylamide and thick gels require an increased period for fixation, and overnight soaking is recommended.
7. The staining time is not critical and staining can be performed overnight without gels overdeveloping. In this case, the containers used for staining should be wrapped with aluminium foil to avoid light exposure.

8. Gels stained with SYPRO Ruby can be stored in the washing solution for a period of time (several days to a week) before image acquisition. In this case, the containers used for staining should be wrapped with aluminium foil to avoid light exposure.
9. SYPRO Ruby dye can be excited using a standard 300 nm UV transilluminator, so that gel images can be documented using any suitable camera system. However, for quantitative analysis the gel must be digitised using a suitable imaging system such as laser fluorimager (equipped with 450, 473, 488 or even 532 nm lasers) or a multi-wavelength CCD-based fluorimaging system ***(28).***

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Detection of Glycoproteins in Gels and Blots

Nicolle H. Packer, Malcolm S. Ball, Peter L. Devine, and Wayne F. Patton

1. Introduction

As we become more aware of the significance of posttranslational modifications, such as glycosylation, in the production of recombinant proteins and in the proteomic studies of development and disease, techniques for the identification and characterization of the oligosaccharides attached to proteins need to be established.

After separation of the proteins by either one-diemensional-(1D)- or two-dimensional-(2D)-polyacrylamide gel electrophoresis (PAGE), the initial step is to identify which proteins are glycoproteins so that further characterization can proceed. Various methods have been developed since the early detection of glycoproteins on gels ***(1)*** and blots, with color, fluorescence, and lectin detection now carried out at the analytical level. The actual level of detection of course depends on the extent of glycosylation of the protein, as the reagents react only with the carbohydrate moiety. We have chosen to describe here the stains that we have found to be the most useful for visualizing glycoproteins, both on gels and blots, after separation by electrophoresis. It should be noted that all the staining procedures currently in use destroy the structure of the carbohydrate and thus prevent further analysis of the glycan component once the glycoprotein is visualized.

As an initial characterization step, once the glycoprotein of interest has been located, a protocol for analyzing the monosaccharide composition on replicate blots of these proteins is also described.

1. Periodic acid-Schiff staining is a generally useful technique for locating glycoproteins on gels and nitrocellulose blots, although the sensitivity may not be sufficient for many applications. Realistically, only 1–10 μg of a highly glycosylated protein

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can be detected and the stain is most useful for mucins and proteoglycans. Periodic acid oxidizes vicinal diols of glycosyl residues to dialdehydes. The aldehydes are then allowed to react with fuchsin (Schiff's reagent) to form a Schiff base. Glycoproteins stain pink with fuchsin on a clear background.

2. Digoxigenin (DIG)–anti-digoxigenin, alkaline phosphate (AP) labeling is an extension of the periodic acid–Schiff method above although the sensitivity is much greater (realistically, depending on the degree of glycosylation, about 0.1 μg of glycoprotein). Glycoproteins can be detected on dot blots or after Western transfer to membranes such as nitrocellulose or polyvinylidene difluoride (PVDF). Vicinal (adjacent) hydroxyl groups in sugars of glycoconjugates are oxidized to aldehyde groups by mild periodate treatment. The spacer-linked steroid hapten digoxigenin (DIG) is then covalently attached to these aldehydes via a hydrazide group. DIG-labeled glycoconjugates are subsequently detected in an enzyme immunoassay using a DIG-specific antibody conjugated with AP. DIG glycan detection is known to label almost all known *N*- and *O*-linked glycans including GPI anchors.
3. Lectins are carbohydrate binding proteins which are particularly useful in glycoprotein and carbohydrate analysis as they can be conjugated to a variety of enzymes or haptens for use in sensitive detection systems. Their specificity can be used to probe for specific structures in the glycoconjugates. Lectins are usually classified on the basis of the monosaccharides with which they interact best, but it is important to note that complex glycoconjugates are generally found to be much better ligands. In addition, the position of a particular monosaccharide in a glycan chain (i.e., to what it is attached) will affect lectin binding, so results obtained in lectin binding studies should be treated with caution. For example, (a) the wheat germ agglutin (WGA) is inhibited most strongly by dimeric GlcNAc, but in glycoproteins this lectin also reacts very strongly with sialic acid and peptide-linked GalNAc; (b) the peanut agglutin binds to Gα1,3GalNAc but does not react when this structure is sialylated. The labeled lectin–carbohydrate conjugate can then be visualized by enzyme immunoassay in the same way as in (b).
4. Recently a new kit that uses a fluorescent hydrazide to react with the periodate-oxidized carbohydrate groups on glycoproteins has been released commercially. The fluorescent tag (Pro-Q™ Emerald 300) is excited by ultraviolet light and emits at a visible light (green) wavelength. The fluorescent signal allows an increased level of detection of the glycoproteins (down to 1 ng of protein) on both gels and PVDF blots, while allowing the subsequent visualization of the total proteins with another fluorescent stain emitting at a different wavelength.
5. The monosaccharide composition of a glycoprotein is a useful start to full characterization. This is obtained by hydrolysis of the separated glycoprotein spots that have been electroblotted to PVDF, followed by monosaccharide analysis using high pressure anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) *(2)*.

In all methods, it is useful to include a glycosylated protein such as transferrin or ovalbumin in the marker lane as a control.

2. Materials

2.1. Periodic Acid-Schiff Staining

1. Solution A: 1.0% (v/v) periodic acid in 3% acetic acid. Periodic acid is corrosive and volatile—**handle with caution**. Be aware of the concentration of periodic acid in the solution that is being diluted, as periodic acid is only about 50% out of the reagent bottle.
2. Solution B: 0.1% (w/v) Sodium metabisulfite in 10 m*M* HCl.
3. Schiff's reagent: A commercial reagent from Sigma Chemical Co. (St. Louis, MO, USA) may be used or better staining can often be achieved by making fresh reagent:
   a. Dissolve 1 g of basic fuchsin in 200 mL of boiling distilled water, stir for 5 min and cool to 50°C.
   b. Filter and add 20 mL of 1 *M* HCl to filtrate.
   c. Cool to 25°C, add 1 g of potassium metabisulfite, and leave to stand in the dark for 24 h.
   d. Add 2 g of activated charcoal, shake for 1 min, and filter. Store at room temperature in the dark.

   Schiff's reagent is corrosive and slightly toxic and a very dilute solution will stain anything with oxidized carbohydrates a pink-purple color **Note:** wear gloves and protective clothing when using this solution and washing it out of the gel/blot.
4. Solution C: 50% (v/v) Ethanol.
5. Solution D: 0.5% (w/v) Sodium metabisulfite in 10 m*M* HCl.
6. Solution E: 7.5% (v/v) Acetic acid–5% (v/v) methanol in distilled water.

   Solutions A, B, and D should be made up freshly.

2.2. DIG–Anti-DIG AP Labeling

1. Buffer A (TBS): 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
2. Buffer B: 100 m*M* Sodium acetate, pH 5.5.
3. Buffer C: 100 m*M* Tris-HCl, 50 m*M* $MgCl_2$, 100 m*M* NaCl.
4. Buffer D: 250 m*M* Tris-HCl, pH 6.8, 8% (w/v) sodium dodecyl sulfate (SDS), 40% (v/v) glycerol, 20% (v/v) 2-mercaptoethanol, bromophenol blue as tracking dye.
5. Buffer E: 50 m*M* potassium phosphate, 150 m*M* NaCl, pH 6.5 (PBS).
6. Blocking reagent: A fraction of milk proteins that are low in glycoproteins. 0.5 g sample is dissolved in 100 mL of buffer A. The solution should be heated to 60°C with stirring; the solution will remain turbid. Allow the solution to cool before immersing the membrane.
7. DIG Glycan Detection Kit (Roche Diagnostics, Basel, Switzerland) containing:
   a. Solution 1: 10 m*M* sodium metaperiodate in buffer B.
   b. Solution 2: 3.3 mg/mL of sodium metabisulfite.
   c. DIG-succinyl-ε-amidocaploic acid hydrazide.
   d. Anti-DIG-AP: Polyclonal sheep anti-DIG Fab fragments, conjugated with AP (750 U/mL).
   e. Solution 3: 75 mg/mL 4-nitroblue tetrazolium chloride dissolved in 70% (v/v) dimethylformamide.

   f. Solution 4: 50 mg/mL of 5-bromo-4-chloro-3-indolyl-phosphate dissolved in dimethyl-formamide.

Make sure that solutions 3 and 4 are still good, as after a few weeks the solutions may begin to precipitate thus reducing the staining efficiency. Store these solutions (3 and 4) in the dark.

2.3. DIG-Labeled Lectin Staining

1. 1% KOH.
2. Blocking reagent (Roche Diagnostics): A fraction of milk proteins that are low in glycoproteins. A 0.5 g sample is dissolved in 100 mL of TBS. The solution should be heated to 60°C with stirring; the solution will remain turbid. Allow the solution to cool before immersing the membrane. Other blockers such as skim milk powder, gelatin, or bovine serum albumin (BSA) may lead to high background.
3. TBS: 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
4. TBS-Tween: TBS + 0.05% Tween 20.
5. Divalent cation stock solution: 0.1 *M* $CaCl_2$, 0.1 *M* $MgCl_2$, 0.1 *M* $MnCl_2$.
6. DIG-labeled lectins (Roche Diagnostics): Sambucus sieboldiana agglutinin (SNA), Maackia amurensis agglutinin (MAA), Arachis hypogaea (peanut) agglutinin (PNA), and Datura stramonium agglutinin (DSA).
7. Part of DIG Glycan Detection Kit (Roche Diagnostics) comprising:
   a. Anti-DIG-AP: Polyclonal sheep anti-DIG Fab fragments, conjugated with AP (750 U/mL)
   b. Buffer C: Tris-100 m*M* HCl, 50 m*M* $MgCl_2$, 100 m*M* NaCl.
   c. Solution 3: 75 mg/mL of 4-nitrobluetetrazolium chloride dissolved in 70% (v/v) dimethylformamide.
   d. Solution 4: 50 mg/mL of 5-bromo-4-chloro-3-indolyl-phosphate dissolved in dimethylformamide.

   Store these solutions (3 and 4) in the dark.

2.4. Pro-Q Emerald 300 Dye Staining

1. Pro-Q Emerald 300 glycoprotein gel stain kit (Molecular Probes, OR, USA) containing:
   a. Pro-Q Emerald 300 reagent, 50× concentrate in dimethyl formamide (DMF) (component A), 5 mL.
   b. Pro-Q Emerald 300 dilution buffer (component B), 250 mL.
   c. Periodic acid (component C), 2.5 g.
   d. SYPRO Ruby protein gel stain (component D), 500 µL.
   e. SDS, component E), 500 µL of a 10% solution.
   f. CandyCane glycoprotein molecular weight standards (component F), 40 µL, sufficient volume for approx 20 gel lanes. Each protein is present at approx 0.5 mg/mL. The standards contain a mixture of glycosylated and nonglycosylated proteins, which, when separated by electrophoresis, provide alternating positive and negative controls.

2. Fix solution: Prepare a solution of 50% methanol and 50% dH_2O. One 8 × 10 gel will require approx 100 mL of fix solution.
3. Wash solution. Prepare a solution of 3% glacial acetic acid in dH_2O. One 8 × 10 gel will require about approx 250 mL of wash solution.
4. Oxidizing solution. Add 250 mL of 3% acetic acid to the bottle containing the periodic acid (component C) and mix until completely dissolved.
5. CandyCane molecular weight standards diluted in sample buffer. For a standard lane on a 8 cm × 10 cm gel, dilute 0.5 µL of the standards with 7.5 µL of sample buffer and vortex-mix. This will result in approx 250 ng of each protein per lane, a sufficient amount for detection of the glycoproteins by the Pro-Q Emerald 300 stain. For large 16 cm × 18 cm gels, double the amount of standard and buffer used.

2.5. HPAEC Analysis of Monosaccharide Composition

1. Methanol.
2. 0.1 *M* TFA.
3. 2 *M* TFA.
4. Standard sugars:
   a. 0.1 mmole/mL of lactobionic acid.
   b. 0.1 mmole/mL of 2-deoxyglucose.
   c. 0.1 mmole/mL of *N*-acetyl neuraminic acid and *N*-glycolyl neuraminic acid.
   d. 0.1 mmole/mL of mixture of fucose, 2-deoxyglucose, galactosamine, glucosamine, galactose, glucose and mannose.

   Sugars should be dried thoroughly over phosphorus pentoxide in a desiccator before weighing.
5. Metal-free HPLC System with DIONEX CarboPac PA1 PA10 column, 4 mm × 25 cm and pulsed amperometric detector (HPAEC-PAD).

3. Methods

3.1. Periodic Acid–Schiff Staining

This method is essentially as described in **ref. *3*** for glycoproteins transferred to nitrocellulose membranes. A modification of the method for PVDF membranes is described in **ref. *4***.

3.1.1. Gel Staining (see **Note 1***)*

1. Soak the gel in solution C for 30 min (*see* **Note 2**).
2. Wash in distilled water for 10 min. All of the ethanol must be removed from the gel, so make sure that the gel is immersed in the water properly. If necessary wash a second time to ensure the removal of the ethanol.
3. Incubate in solution A for 30 min. **Beware of the fumes from the acid.** From this point onwards the gel should be placed in the fume hood.
4. Wash in distilled water for at least 6 × 5 min or 5 × 5 min and 1× overnight.
5. Wash in solution B for 2 × 10 min. At this stage make up 100 mL of solution B and perform 2 × 30 mL washes; save the final 40 mL for **step 7**.

6. Incubate in Schiff's reagent for 1 h in the dark. It is essential after adding the Schiff's reagent that the gel is kept in the dark, as any light will stop the color from developing.
7. Incubate in solution B for 1 h in the dark.
8. Wash several times in solution D for a total of at least 2 h and leave as long as overnight to ensure good color detection (*see* **Notes 3** and **4**).
9. Store the gel in solution E.

3.1.2. Membrane Staining (see **Note 5***)*

1. Wash the membrane in distilled water for 5 min (*see* **Note 6**).
2. Incubate in solution A for 30 min.
3. Wash in distilled water for 2 × 5 min.
4. Wash in solution B for 2 × 5 min.
5. Incubate for 15 min in Schiff's reagent (*see* **Note 3**).
6. Wash in solution B for 2 × 5 min.
7. Air dry the membrane.

3.2. DIG–Anti-DIG AP Labeling (See Note 7)

This staining procedure can only be used on membranes; however, the proteins can be prelabeled in solution before electrophoresis, or labeled on the membrane after blotting (*see* **Notes 8** and **9**). In both cases the color development is the same. Nitrocellulose membranes can be used but some background staining can occur with postlabeling. In preference, the proteins should be blotted onto PVDF.

The reagents for this method are provided in the Roche Diagnostics DIG Glycan Detection Kit and the methods described are essentially taken from that kit.

A similar kit based on biotin/streptavidin binding instead of the DIG–Anti-DIG interaction is marketed by Bio-Rad as the Immun-Blot® Glycoprotein Detection Kit.

3.2.1. Prelabeling (see **Note 8***)*

1. Dilute protein solution 1:1 to 20 µL with buffer B (*see* **Note 7**).
2. Add 10 µL of solution 1 and incubate for 20 min in the dark at room temperature.
3. Add 10 µL of solution 2 and leave for 5 min. The addition of the sodium bisulfite destroys the excess periodate.
4. Add 5 µL of DIG-succinyl-e-amidocaproic acid hydrazide, mix, and incubate at room temperature for 1 h. Sensitivity may be increased by increasing the incubation time to several hours.
5. Add 15 µL buffer D and heat the mixture to 100°C for 5 min to stop the labeling.
6. Separate the labeled glycoproteins by SDS-PAGE and blot to the membrane (*see* **Note 10**) which is now ready for the staining reaction (*see* **Subheading 3.2.3**).

3.2.2. Postlabeling (see **Note 9***)*

1. Wash the membrane for 10 min in 50 mL buffer E (PBS). (*see* **Note 11**).
2. Incubate the membrane in 20 mL of solution 1 for 20 min at room temperature. For low amounts of oligosaccharide it may be necessary to increase the amount of sodium metaperiodate in the solution. Increasing the concentration up to 200 m*M* increases the final staining.
3. Wash in buffer E, 3 × 10 min
4. Incubate the membrane in 5 mL of buffer B containing 1 mL of DIG-succinyl-ε-amidocaproic acid hydrazide for 1 h at room temperature. For low amounts of glycoproteins greater sensitivity can be obtained by increasing the concentration of DIG-succinyl-ε-amidocaproic acid hydrazide; however no further benefit is gained by raising the concentration greater than 3 mL in 5 mL (*see* **Note 12**).
5. Wash for 3 × 10 min in buffer A. TBS may now be used to wash the membrane as the DIG labeling has taken place.

3.2.3. DIG Staining Reaction

1. Incubate the membrane for at least 30 min in the blocking reagent (*see* **Note 10**). The membrane can be stored for several days at 4°C at this stage, and in fact a lower background staining can be achieved by allowing the filter membrane to wash in the solution at 4°C overnight (shaking is not necessary) and then for 30 min at room temperature with shaking.
2. Wash for 3 × 10 min in buffer A.
3. Incubate the membrane with 10 mL of buffer A containing 10 µL of anti-digoxigenin-AP at room temperature for 1 h. Sensitivity can be increased by increasing the amount of anti-DIG in the solution by a factor of 2 (although any more has no appreciable effect), or by increasing the incubation time to several hours.
4. Wash for 3 × 10 min with buffer A.
5. Immerse the membrane without shaking into 10 mL of buffer C containing 37.5 µL solution 4 and 50 µL solution 3 (mix just before use). If there is a large amount of sugar present in the bands the color reaction can take only a few minutes; however, if there is little material the reaction could take several hours or overnight. The reaction is best done in the dark, as light can cause nonspecific staining of the membrane. If solutions 3 and 4 are not fresh then the reaction can take several hours and cause a large amount of background staining. It is possible to speed up the reaction by doubling the amounts of solutions 3 and 4 added to buffer C.
6. Wash the membrane several times with Milli-Q water and allow to air dry. The membrane is best stored in foil to reduce fading of the bands once the reaction is stopped.

3.3. DIG - Labeled Lectin Staining

A wide range of lectins are commercially available as free lectin or conjugates of peroxidase, biotin, DIG, fluorescein isothiocyanate (FITC), alcohol dehydrogenase, colloidal gold, or on solid supports such as agarose. A list of commonly used commercially available lectins is shown in **Table 1**. Peroxidase

Table 1
Some Commonly Used Lectins[a]

Taxonomic name	Common name	Specificity[b,c]
Aleuria aurantia	AAA	α-(1,6)Fuc
Amanranthus caudatus	Amaranthin (ACA)	Galb-(1,3)GalNAc
Canavalia ensiformis	Jackbean concancavalin A	α-Man > α-Glc (Con A)
Datura stramonium	Jimson weed (DSA)	β-(1,4)GlcNAc Terminal GlcNAc
Maackia amurensis	MAA	α-(2,3)Neu-N-Ac
Phaseolis vulgaris	Red kidney bean (PHA-L)	complex *N*-linked
Arachis hypogaea	Peanut (PNA)	Gal (1,3)GalNAc
Ricinus communis	Castor bean	Terminal Gal (RCA120)
Sambucus sieboldiana	Elderberry (SNA)	α-(2,6)Neu-N-Ac
Triticum vulgaris	Wheat germ (WGA)	$(GlcNAc)_2$ > GlcNAc
Helix pomatia	Snail, edible (HPA)	Terminal GalNAc
Lens culinaris	Lentil	α-Man, α-Glc
Glycine max	Soybean (SBA)	GalNAc
Erythrina cristagalli	Coral tree (ECA)	Gal (1,4)GlcNAc

[a] Many lectins are available commercially as free (unlabeled); digoxigenin (DIG) labeled; biotinylated; peroxidase labelled; or conjugated to agarose beads from companies such as Roche Diagnostics, Sigma Chemical Company, and Amersham-Pharmacia.

[b] GalNAc, *N*-acetyl galactosamine; GlcNAc, *N*-acetylglucosamine; Gal, galactose; Glc, glucose; Man, mannose; Fuc, fucose; NeuNAc, *N*-acetylneuraminic acid (sialic acid).

[c] D-sugars are the preferred sugars.

or AP-labeled lectins can be detected directly. Alternatively, lectins can be detected using anti-DIG peroxidase (if DIG labeled) or streptavidin peroxidase (if biotin labeled), followed by an insoluble substrate. Sensitivity is generally increased with these indirect methods. The detection can be carried out with blots on nitrocellulose or PVDF (*see* **Note 13**).

1. Fix membrane for 5 min with 1% KOH. (*See* **Note 12**).
2. Rinse for 1 min with distilled water.
3. Block unbound sites with a 1-h incubation at room temperature with blocking reagent.
4. Rinse away blocking reagent with 3 × 1 min washes with TBS-Tween.
5. Add DIG-labeled lectins diluted in TBS-Tween containing 0.1 m*M* $CaCl_2$, 0.1 m*M* $MgCl_2$, 0.1 m*M* $MnCl_2$. Leave overnight at 4°C (*see* **Notes 14** and **15**).
6. Remove unbound lectin by washing with 6 × 5 min in TBS-Tween.
7. Incubate nitrocellulose with 10 µL of anti-DIG AP diluted in 10 µL of TBS for 1 h at room temperature.
8. Repeat washing **step 6**.

9. Immerse the membrane without shaking into 10 µL of buffer C containing 37.5 µL of solution 4 and 50 µL of solution 3 (mix just before use).
10. Stop reaction when gray to black spots are seen (few minutes to overnight) by rinsing with water.

3.4. Pro-Q Emerald 300 Dye Staining

Pro-Q™ Emerald 300 Glycoprotein Gel Stain Kit (Molecular Probes, OR) provides a method for differentially staining glycosylated and nonglycosylated proteins in the same gel. The technique combines the green fluorescent Pro-Q™ Emerald 300 glycoprotein stain with the red fluorescent SYPRO Ruby total protein gel stain. A related product, Pro-Q™ Emerald 300 Glycoprotein Blot Stain Kit, allows similar capabilities for proteins electroblotted to PVDF membranes.

Using this stain allows detection of <1 ng of glycoprotein/band, depending on the nature and the degree of glycosylation, making it 500-fold more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye. The green fluorescent signal from Pro-Q Emerald 300 stain can be visualized with 300 nm UV illumination.

1. Separate proteins by standard SDS-PAGE. Typically, the sample is diluted to about 10–100 µg/mL with sample buffer and 5–10 µL of diluted sample is added per lane for 8 cm × 10 cm gels. Large, 16 cm × 18 cm gels require twice as much material.
2. After electrophoresis, fix the gel by immersing it in 75–100 mL of fix solution and incubating at room temperature with gentle agitation (e.g., on an orbital shaker at 50 rpm) for 45 min.
3. Wash the gel by incubating it in 50 mL of wash solution with gentle agitation for 10 min. Repeat this step once.
4. Incubate the gel in 25 mL of oxidizing solution with gentle agitation for 30 min.
5. Wash the gel in 50 mL of wash solution with gentle agitation for 5–10 min. Repeat this step twice more.
6. Prepare fresh Pro-Q Emerald 300 Staining Solution by diluting the Pro-Q Emerald 300 reagent (component A) 50-fold into Pro-Q Emerald 300 dilution buffer (component B). For example, dilute 500 µL of Pro-Q Emerald 300 reagent into 25 mL of dilution buffer to make enough staining solution for one 8 × 10 cm gel.
7. Incubate the gel in the dark in 25 mL of Pro-Q Emerald 300 Staining Solution while gently agitating for 90–120 min. The signal can be seen after about 20 min and maximum sensitivity is reached at about 120 min. Staining overnight is not recommended.
8. Wash the gel with 50 mL of wash solution at room temperature for 15 min. Repeat this wash once for a total of two washes. Do not leave the gel in wash solution for more than 2 h, as the staining signal will start to decrease.
9. Visualize the stain using a standard UV transilluminator (*see* **Notes 16–20**).
10. To counter-stain nonglycosylated proteins in the sample, completely thaw the 10% SDS solution (component F), vortex-mixing to completely dissolve the SDS.

11. Dilute the 10% SDS solution 2000-fold into the SYPRO Ruby protein gel stain to make a final concentration of 0.005% SDS.
12. Proceed with staining as outlined in Chapter 49 in this volume describing the SYPRO Ruby protein gel staining procedure (*see* **Note 21**).

3.5. HPAEC Analysis of Monosaccharide Composition (See Note 22)

This technique requires about 100 pmol (5 µg) of glycosylated protein.

1. Excise spots that have been visualized by coomassie blue or amido black on PVDF membranes and place into screw-capped Eppendorf tubes (*see* **Note 23**).
2. Wet membrane with methanol. If the membrane is not properly wetted it will float and the hydrolysis will not be complete.

3.5.1. Analysis of Sialic Acids

1. Add 100 µL 0.1 *M* TFA to the wetted membrane. Mix and close the tube.
2. Incubate in heating block at 80°C for 40 min.
3. Remove membrane from tube and wash with 50 µL of water.
4. Dry combined solutions in a Speed Vac concentrator.
5. Resuspend in 25 µL and add 0.5 nmol lactobionic acid as internal standard for quantitation. Analyze by HPAEC-PAD using a linear gradient of 0–200 m*M* NaAcetate in 250 m*M* NaOH for 30 min (*see* **Notes 24–26**).
6. Compare retention time with standard of 1 nmol *N*-acetyl and *N*-glycolylneuraminic acids.

3.5.2. Analysis of Neutral Monosaccharides

1. Add 100 µL of 2 *M* TFA to the desialylated membrane spots (*see* **Note 27**). Mix and close tube (*see* **Note 24**). Re-wet membrane with methanol before addition of acid if dried out.
2. Incubate in a boiling water bath for 4 h.
3. Remove membrane from tube and wash with 50 µL of water. Keep membrane for subsequent amino acid analysis if required.
4. Dry combined solutions in a Speed Vac concentrator.
5. Resuspend in 25 µl of water and add 0.25 nmol of 2-deoxyglucose as internal standard for quantitation. Analyze by HPAEC-PAD eluted isocratically with 12 m*M* NaOH for 30 min (*see* **Notes 24–26**).
6. Compare with a standard mixture containing fucose, 2-deoxyglucose, galactosamine, glucosamine, galactose, glucose, and mannose (5 µl of 1 m*M* solution) (*see* **Note 28**). This is the order of elution of the monosaccharides from the CarboPac column.

4. Notes

4.1. Periodic Acid/Schiff Staining

1. This method can be used on PAGE gels, agarose, or polyacrylamide–agarose composite gels. The procedure should be carried out on an orbital shaker or rocker (an orbital shaker may cause background swirls on some

gels). High background staining can occur when staining some batches of agarose or composite gels.

2. All steps in this procedure should be performed with shaking and should be carried out in a fume hood.
3. A negative result often means that insufficient protein is present or that the protein has little glycosylation
4. Be aware that highly glycosylated proteins do not transfer well to nitrocellulose membranes, and failure to detect may be due to inefficient transfer to the membrane. It is sometimes useful to carry out the stain on the gel after transfer, to test for remaining glycoprotein.
5. PAS staining after transfer of glycoproteins to membranes eliminates the need for extensive fixation steps, as well as shortening the time needed for washing steps, without loss of staining intensity. Results are easier to visualize and are easier to store. Before Western transfer, it is suggested to gently rock the gel for 30–60 min in transfer buffer so as to remove SDS, which can lead to higher backgrounds during membrane staining.
6. Periodic acid–Schiff staining on nitrocellulose membranes increases sensitivity slightly and reduces washing times considerably compared with gel staining.

4.2. DIG–Anti-DIG AP Labeling

7. All steps in the procedure except for color development should be done on a shaker or rocker. Swirling background staining may occur when using a orbital shaker.
8. Prelabeling gives a higher sensitivity than postlabeling, requires less DIG (1 µl per labeling), and can be used with <0.25% SDS, Nonidet P-40 (NP-40), Triton X-100, but not with octylglucoside. Prelabeling results in broader bands than post-labeling and may result in a change of p*I* of the proteins in the first dimension. Pre-labeling is negatively influenced by mercaptoethanol, dithiothreitol (DTT), glycerol and Tris.
9. The postlabeling procedure is used for proteins that have already been separated by gel electrophoresis and immobilized on blots. It is important to use PBS (buffer E) instead of TBS (buffer A) in the initial stages as Tris inhibits the DIG labeling process.
10. Nitrocellulose and PVDF membranes can be used. Nylon membranes result in high background.
11. For mucins and other heavily glycosylated proteins it may be necessary to fix the bands to the membrane before labeling by washing in 1% KOH for 5 min. If this procedure is done, increase the initial washes to 3 × 10 min in buffer B.
12. Postlabeling results in sharper bands than prelabeling but has a lower sensitivity and requires more DIG (5 µL per gel).

4.3. DIG-Labeled Lectin Staining

13. The binding of some lectins will be increased by desialylation while the binding of others will be decreased.
14. Suggested dilutions of the DIG labeled lectins: SNA—1:1000, MAA—1:500, DSA—1:1000, PNA—1:100.
15. The divalent ions are necessary for optimal lectin reactivity. The stock solution should be diluted to give a final concentration of 1 m*M* of each ion.

4.4. Pro-Q Emerald 300 Dye Staining

16. The Pro-Q Emerald 300 stain has an excitation maximum at approx 280 nm and an emission maximum near 530 nm. Stained glycoproteins can be visualized using a 300 nm UV transilluminator. *The use of a photographic camera or charge coupled device (CCD) camera and the appropriate filters is essential to obtain the greatest sensitivity*.
17. It is important to clean the surface of the transilluminator after each use with deionized water and a soft cloth (such as cheesecloth). Otherwise, fluorescent dyes can accumulate on the glass surface and cause a high background fluorescence.
18. Use a 300 nm transilluminator with six 15 W watt bulbs. Excitation with different light sources may not give the same sensitivity.
19. Using a Polaroid® camera and Polaroid 667 black-and-white print film, the highest sensitivity is achieved with a 490 nm longpass filter, such as the SYPRO protein gel stain photographic filter (S-6656), available from Molecular Probes. Gels are typically photographed using an f-stop of 4.5 for 2–4 s, using multiple 1-s exposures.
20. Using a CCD camera, images are best obtained by digitizing at about 1024 × 1024 pixels resolution with 12–, 14– or 16– bit gray scale levels per pixel. A 520 nm long pass filter is suitable for visualizing the stain. A CCD camera-based image analysis system can gather quantitative information that will allow comparison of fluorescence intensities between different bands or spots. Using such a system, the Pro-Q Emerald stain has a linear dynamic range over three orders of magnitude. The polyester backing on some premade gels is highly fluorescent. For maximum sensitivity using a UV transilluminator, these gels should be placed polyacrylamide side down and an emission filter used to screen out the blue fluorescence of the plastic.
21. The green-fluorescent Pro-Q Emerald 300 staining should be viewed and documented before staining total proteins with SYPRO Ruby protein gel stain. Pro-Q Emerald dye signal will fade somewhat after SYPRO Ruby dye staining.

4.5. HPAEC Analysis of Monosaccharide Composition

22. Data on the composition of both the acidic sialic acids and the hexoses and amino sugars can be obtained sequentially from a single spot. After the

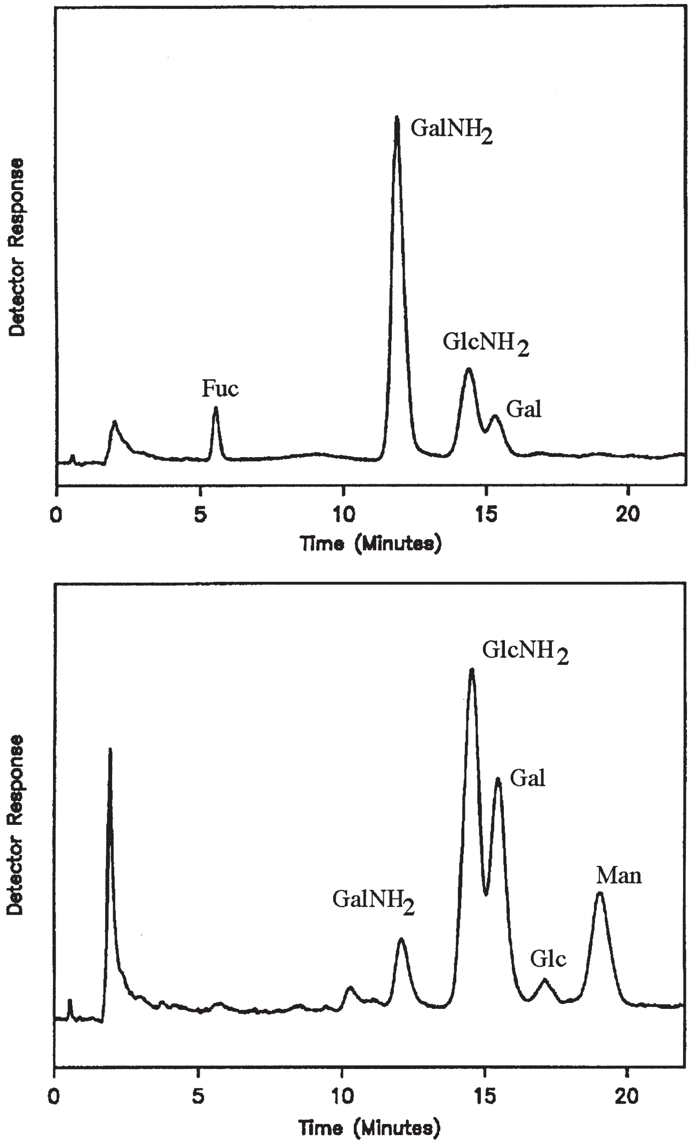

Fig. 1. Monosaccharide composition of glycoprotein hydrolysates. *(Upper)*, Bovine submaxillary mucin; *(lower)*, fetuin. Separation by HPAEC-PAD.

4 *M* TFA hydrolysis, amino acid analysis (6 *M* HCl, 1 h, 155°C) can be performed subsequently on the same spot for identification of the protein.

23. It is important for monosaccharide analysis that the tubes be kept clean in a cellulose-free environment (i.e., no paper, cotton wool, or dust!) because of the common glucose contamination from the hydrolysis of cellulose

and other ubiquitous polysaccharides. For the same reason a blank of all reagents should be run to establish background contamination levels.

24. The HPLC system must be totally metal free because of the caustic reagents. All solvents must be made free of carbon dioxide by boiling of the MilliQ water used. The water is cooled under argon and the solutions kept under helium to reduce the precipitation of sodium carbonate from the sodium hydroxide solutions and the resultant effect on the anion-exchange chromatography.
25. The DIONEX CarboPac PA10 column gives better separation of the monosaccharides than the CarboPac PA1 but the latter may still be used effectively and can be used for both monosaccharide and oligosaccharide analysis.
26. The columns must be washed with strong alkali (0.4 *M* NaOH) for 10 min and re-equilibrated between injections.
27. If more accurate quantitation of the amino sugars is required, stronger hydrolysis using 4 *M* HCl for 4 h at 100°C can be carried out instead of the milder 4 *M* TFA hydrolysis. Subsequent amino acid analysis cannot be performed in this case.
28. Glucose will almost always be present as a contaminant and so is exceptionally difficult to quantitate if it is a constituent. Examples of the monosaccharide composition of typical *N*-linked sugars (fetuin) and *O*-linked sugars (mucin) are shown in **Fig. 1**.

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Staining of Glycoproteins/Proteoglycans on SDS-Gels

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1. Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly employed technique for separation of proteins according to size. Among other applications, it is used for identification and characterization of proteins on the basis of molecular weight determinations. In this chapter, staining methods will be described that permit detection of highly glycosylated proteins on SDS gels at levels of a few nanograms.

Proteins with limited glycosylation are most often stained with Coomassie brilliant blue or, if high sensitivity is needed, with a silver-stain ***(1)***. These stains, however, are far less sensitive when used for detecetion of highly glycosylated proteoglycans (protein-glycosaminoglycans) or glycoproteins (protein-oligosaccharides), leading to weak staining or even failure of detection. This is presumably the result of steric interference by the carbohydrates with the binding of silver ions.

Proteoglycans are traditionally stained with cationic dyes, such as alcian blue or toluidine blue ***(2)***, that bind to the negatively charged glycosaminoglycan side chains, whereas more neutral glycoproteins can be detected by some variation of the Schiff base reaction involving initial oxidation of carbohydrates by periodic acid and subsequent staining with Schiff's reagent (PAS) ***(3)***, alcian blue ***(4)***, or a hydrazine derivate ***(5)***. A protocol for PAS staining of small SDS gels is described in **ref. *6***. However, these methods, although useful in many instances, are characterized by low sensitivity, generally requiring microgram amounts of protein for detection. At the same time, they are carbohydrate specific, which means that nonglycosylated proteins are not stained.

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The two methods described here are based on silver enhancement of traditional staining methods for proteoglycan and glycoprotein ***(7–12)***, which result in a twofold increase in sensitivity as compared with alcian blue or PAS alone. In both methods, alcian blue is used as the primary staining agent, binding either directly to the proteoglycans (**Subheading 3.1.**) or to oxidized glycoproteins (**Subheading 3.2.**), subsequently enhanced by a neutral silver-staining protocol.

The methods also stain highly negatively charged phosphoproteins (e.g., osteopontin and bone sialoprotein, rich in phosphate and acidic amino acids) that stain weakly with ordinary methods ***(13)***. Furthermore, both glycosylated and nonglycosylated proteins are stained. Nonglycolylated proteins, however, stain weaker, and it is possible to exclude the staining of nonglycosylated proteins (*see* **Note 1** and **ref. *14***).

A protocol for staining by the cationic dye "Stains-All" instead of alcian blue, combined with silver is described in **ref. *13***. This method seems to more uniformly stain both nonglycosylated and negatively charged proteins

The high sensitivity of the methods make them very suitable for detection of proteoglycan/glycoprotein in dilute mixed samples, and for characterization of small amounts of purified materials, e.g., by determination of molecular weight before and after deglycosylation ***(15–17***). In this connection, it is important to note that, owing to heterogenity of carbohydrate substitution, highly glycosylated proteins usually move as diffuse bands or broader smears, so that unambiguous determination of molecular weight is not always possible.

For more specific purposes, other detection systems are usually employed. Sensitive detection of glycoproteins can be achieved with lectins or specific antibodies, most often after blotting onto a membrane ***(18, 19)***. Proteoglycans can similarly be identified by antibodies directed toward the core protein or glycosaminoglycan structures ***(20, 21***), and glycosaminoglycans can be characterized by mass spectrometry ***(22***).

The methods described here are optimized for the small, supported gels used in the PhastSystem (Pharmacia, Uppsala, Sweden), which can be programmed for automatic staining resulting in fast and reproducible results. If other systems (larger gels) are used, generally longer time is needed in each step (*see* **Note 2**).

2. Materials

2.1. Staining of Proteoglycans

All chemicals used should be of analytical grade, and water should be of high purity.

1. Washing solution I: 25% ethanol (v/v), 10% acetic acid (v/v) in water.
2. Washing solution II: 10% ethanol (v/v), 5% acetic acid (v/v) in water.
3. Staining solution: 0.125% alcian blue (w/v) in washing solution I. Stir extensively and filtrate before use.

4. Stopping solution: 10% acetic acid (v/v), 10% glycerol (v/v) in water.
5. Developer stock: 2.5% (w/v) sodium carbonate in water.
6. Sensitizing solution: 5% (v/v) glutardialdehyde in water. (Prepare fresh when required.)
7. Silvering solution: 0.4% (w/v) silver nitrate in water. (Prepare fresh when required).
8. Developer: Add formaldehyde to the developer stock to a final concentration of 0.013% (v/v). e.g., add 35 µL of a 37% formaldehyde solution to 100 ml of developer stock, and stir for a few seconds. (Prepare fresh just before use).

2.2. Staining of Glycoproteins

1. Prepare all solutions described in **Subheading 2.1.**
2. Fixing solution: 10% (v/v) trichloroacetic acid in water.
3. Washing solution III: 5% (v/v) acetic acid in water.
4. Oxidizing solution: 1% (w/v) periodic acid in water.
5. Reducing solution: 0.5% (w/v) potassium metabisulfite in water.

3. Methods

3.1. Alcian Blue/Silver Staining of Proteoglycans (see Note 2)

This method stains proteoglycans, glycosaminoglycans, phosphoproteins, non-glycosylated proteins, and some glycoproteins with a high content of negatively charged groups. The method can be varied for specific staining of proteoglycan/glycosaminoglycan (*see* **Note 1**).

1. Immediately after electrophoresis, transfer the gel to the development chamber/staining tray (*see* **Note 3**), and wash the gel three times in washing solution I for 5, 10, and 15 min at 50°C (*see* **Note 4**).
2. Add the staining solution and stain the gel for 15 minutes at 50°C.
3. Wash the gel three times in washing solution I for 1, 4, and 5 min and subsequently twice in washing solution II for 2 and 4 min at 50°C (*see* **Note 5**).
4. Add the sensitizing solution for 6 min at 50°C (*see* **Note 6**).
5. Wash the gel twice in washing solution II for 3 and 5 min, and subsequently in water twice for 2 min at 50°C.
6. Submerge the gel in the silvering solution for 6.5 minutes at 40°C.
7. Wash the gel twice in water for 30 s at 30°C (see **Note 7**).
8. Develop the gel with the freshly prepared developer at room temperature for an initial period of 30 s and subsequently for 4–8 min, depending on the desired sensitivity, background staining, and specificity (*see* **Note 1**).
9. Stop the development and preserve the gel by adding the stopping solution for 5 min (*see* **Note 8**).

3.2. Periodic Acid Oxidation and Subsequent Alcian Blue/Silver Staining of Glycoproteins

Some glycoproteins stain weakly with alcian blue/silver alone, probably owing to the low content of negatively charged groups for binding of alcian blue. This is overcome by initial oxidation of carbohydrates by periodic acid.

1. After electrophoresis, transfer the gel to the development chamber/Petri dish, and add the fixing solution for 10 min at 30°C (*see* **Note 9**).
2. Wash the gel twice for 2 min in washing solution III and submerge the gel in the oxidizing solution for 20 min at 30°C.
3. Wash the gel twice for 2 min in washing solution III and subsequently in water twice for 2 min before adding the reducing solution for 12 min at 30°C.
4. Wash the gel with water twice for 2 min and subsequently with washing solution I twice for 2 min at 50°C.
5. Continue with **steps 2–9** of **Subheading 3.1** exactly as described.

4. Notes

1. The development time can be varied according to the desired sensitivity and background staining. Even if an automatic staining chamber is used, these steps can be performed advantagously in staining trays/Petri dishes. In the first development step, a dark precipitate is created and the gel is transfered to a fresh developing solution. After 1–3 minutes proteoglycans will appear, whereas nonglycosylated proteins appear after 4–8 minutes. The gel can be photographed sequentially on a light box during the development step for identification of proteoglycans. As a control, a gel can be stained for proteins with ordinary silver (**Subheading 3.1., steps 3–9**), in which proteoglycans will not stain.
2. The methods are optimized for the PhastSystem, which uses small, supported gels that are stained in an automatic development chamber. If larger gels are stained in staining trays at room temperature, longer incubation times are needed. For unsupported 1-mm thick gels, good results are obtained by increasing the washing with washing solution I in **steps 1** and **3** (**Subheading 3.1**) to a total of 2.5 and 1.5 hours, respectively, with four changes of the solution, and furthermore doubling the incubation times in all other steps.
3. Handle gels with gloves or forcepts, since fingerprints stain. The staining methods are highly sensitive, and it is essential that the equipment (development chamber/staining trays) is scrupulously clean and that high-quality water is used. Use a separate tray for the staining solution. Gels should be agitated during all staining, and washing procedures.
4. This rather extensive washing procedure is necessary to remove the SDS from the gel, which otherwise precipitates alcian blue, resulting in excessively high background. If gels are run in native PAGE, one short washing step is sufficient.
5. Alcian blue is irreversibly fixed in the gel by the subsequent silver staining and results in a greenish-black background if the stain is not washed out by dilute acetic acid. Only a weak bluish nuance in the background should remain.
6. Glutaraldehyde is injurious to health. If not carried out in a closed chamber, a fume cupboard should be used.

7. This step is important for washing out excess silver ions without losing silver bound to alcian blue/proteoglycan for autocatalytic reduction in the development step. Too little washing leads to formation of metallic silver in the background, whereas too intense washing leads to decreased sensitivity.
8. The image is stable, but over time, increased background staining will develop, especially from light exposure.
9. In this staining procedure, acetic acid/ethanol solutions are not always efficient for fixation of the proteins in the gel, whereas trichloroacetic acid works well.

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Detection of Proteins and Sialoglycoproteins in Polyacrylamide Gels Using Eosin Y Stain

Fan Lin and Gary E. Wise

1. Introduction

A rapid, sensitive, and reliable staining technique is essential in detection of proteins in polyacrylamide gels. Coomassie brilliant blue R-250 (CBB) is the stain that meets these criteria except for sensitivity; i.e., CBB staining requires relatively large amounts of proteins. It has been reported that the sensitivity for CBB stain in polyacrylamide gels is 0.1–0.5 µg/protein band ***(1)***. This problem of relatively low staining sensitivity is often circumvented by employing silver staining techniques ***(2–5)***. However, it is difficult to transfer silver stained proteins to transfer membranes unless they either are negatively stained by silver ***(6)*** or the positively silver-stained proteins are treated with 2X SDS sample buffer prior to transfer ***(7)***. In addition, sialoglycoproteins cannot be detected by CBB and thus have to be visualized by other stains, such as the periodic acid-Schiff (PAS) reagent ***(8)***, silver stains ***(9)***, or silver/Coomassie blue R-250 double staining technique ***(10)***.

To circumvent the deficiencies of the above staining techniques, we have developed an eosin Y staining technique ***(11)***. This staining method allows one to detect proteins more rapidly than most CBB and silver staining methods. It detects a variety of proteins in amounts as little as 10 ng in polyacrylamide gels, including membrane sialoglycoproteins, and has the added advantage of the antigenicity of the stained proteins being retained. The precise mechanism by which eosin Y stains both proteins and sialoglycoproteins is not fully understood.

However, in the staining protocol described here, the fixing and developing solution of 10% acetic acid/40% methanol (pH 2.5) is strongly acidic. Under such conditions, eosin Y might be converted into its precursor form of the dihydrofluoran. Thus, a protein might be stained by means of both hydrophobic

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interaction between aromatic rings of eosin Y and hydrophobic sites of the protein and by hydrogen bonding between hydroxyl groups of eosin Y and the backbone of a protein. Here we describe this detailed staining protocol and technical advice in order to enable others to obtain optimal staining results.

2. Materials

1. Eosin Y staining solution: A stock solution of 10% eosin Y (w/v) is prepared. This solution is stable at room temperature for at least 6 mo. Each 100 mL of staining solution contains 10 mL of 10% eosin Y solution, 40 mL of 100% methanol, 49.5 mL distilled deionized water, and 0.4–0.5 mL of full strength glacial acetic acid. The staining solution is made and filtered prior to use.
2. Gel fixation solution: 10% glacial acetic acid/40% methanol.
3. Gel developing solution: distilled-deionized water, 10% glacial acetic acid/40% methanol.
4. A black plastic board and a transilluminated fluorescent white light box.

3. Methods

3.1. Staining of Various Protein in SDS-PAGE

1. Immediately following electrophoresis, the SDS-polyacrylamide gel containing given proteins is fixed in 5 gel volumes of 10% glacial acetic acid/40% methanol for 10 min at room temperature with shaking and then rinsed with distilled water twice.
2. The gel is immersed with 200 mL (5–6 gel volumes) of the 1% eosin Y staining solution for 15 min at room temperature with shaking.
3. The gel is transferred to a clean glass container, quickly rinsed with distilled water and then washed with distilled water for 3 min (*see* **Note 2**).
4. The stained bands of the gel are developed by placing the gel in 10% acetic acid/40 methanol for about 15 s (*see* **Note 3**).
5. The development is stopped by immersing the gel in distilled water.
6. The gel can be kept in distilled water for at least 1 mo without fading.
7. The stained gel can be viewed either by using transilluminated fluorescent white light or by placing the gel on a black plastic board with top light illumination.

3.2. Staining of Membrane Sialoglycoproteins in SDS-PAGE

1. Immediately following electrophoresis, the SDS-polyacrylamide gel is placed in 200 mL (5–6 gel volumes) eosin Y staining solution for 45 min at room temperature with shaking.
2. The gel is quickly rinsed with distilled water and then washed with distilled water for 3 changes of 5 min each. Protein bands are visualized at the end of washing step (*see* **Note 4**).
3. The gel is then developed in 10% acetic acid/40% methanol for about 2 min.
4. The development of staining is terminated by washing the gel in distilled water twice.
5. The gel can be kept in distilled water for at least 1 mo without fading.

6. The stained gel can be viewed either by using transilluminated fluorescent white light or by placing the gel on a black plastic board with table top light illumination.

4. Notes

1. The 1% eosin Y staining solution needs to be made fresh and filtered prior to use. Eosin Y should be soluble in both water and methanol. Acetic acid must be added last. The final concentration of acetic acid in this staining solution is critical for staining background and sensitivity. The acetic acid should be added to solution with stirring. After adding acetic acid, the staining solution should appear to be cloudy but should not precipitate. If any precipitation occurs, it indicates that the acetic acid concentration is too high.
2. In **step 3** of staining various proteins, some orange precipitation may cover a SDS-gel surface. One may gently clean up the precipitation by wiping off the gel surface with a latex glove or by using Kimwipe tissue, which will reduce the background and improve the staining sensitivity.
3. An appropriate development time will ensure yellow–orange staining bands. Prolonging development in 10% acetic acid/40% methanol often results in yellow–orange bands becoming brown bands which, in turn, will decrease the staining sensitivity.
4. It should be noted that at the end of the washing step, the proteins should appear to be yellow-orange and the sialoglycoproteins appear to be light yellow. If the protein bands are still not visualized at this point, one may prolong the washing step for another 5–10 min.
5. The eosin Y stained gel can be stored in distilled water for at least 1 mo without fading. It is not recommended to dry and store the stained gels because the intensity and resolution of protein bands are greatly decreased.
6. The eosin Y stained proteins in SDS-gels can be transferred to immobilon-P membrane without additional treatment. The antigenicity of a given protein is usually not affected by the eosin Y stain ***(11)***.

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A Modified Pro-Q Diamond Staining Protocol for Phosphoprotein Detection in Polyacrylamide Gels

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1. Introduction

Protein phosphorylation is one of the most common and arguably best studied post-translational modifications. Virtually all key cellular processes are regulated via reversible protein phosphorylation ***(1, 2)***. Identification of candidate regulatory protein kinases, their substrates, and the phosphorylation site are the key steps toward understanding the regulation of cellular processes and their complex network. This challenging area is known as phosphoproteomics ***(3, 4)***. One of the major advances in this area is the introduction of Pro-Q Diamond phosphoprotein stain (Pro-Q DPS)—a fluorescence-linked assay—to detect phosphoproteins at levels as low as 1 ng on a single polyacrylamide gel ***(5, 6)***. The characteristic features of Pro-Q DPS are: (i) binds directly and specifically to the phosphate moiety of phosphoproteins regardless of which amino acid residue is phosphorylated, (ii) is fully compatible with other stains and mass spectrometry, and (iii) has high sensitivity and linearity, ranging from 1 ng to 1 μg, allowing quantitative analysis of phosphoproteins at the global level. Though these properties are indeed desirable to conduct large-scale phosphoproteomics analysis, Pro-Q DPS is not economically attractive. Most importantly, it also lacks a fully optimized protocol to reproduce results from laboratory to laboratory.

To address these concerns, we recently introduced an alternative staining protocol for Pro-Q DPS ***(7)*** modified from previous publications ***(5, 6)*** and recommended manufacturer's protocols (Molecular Probes). In this chapter, we focus on describing the complete protocol of the modified Pro-Q DPS ***(7)***. Two of the major modifications include a three-fold dilution of Pro-Q DPS (directly by diluting the stock) and the use of three-fold less volume of the diluted staining solution than the manufacturer's recommended volume for one large-format

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Table 1
A Modified Pro-Q DPS Protocol for Phosphoprotein Detection in Polyacrylamide Gels

Step	Solution	Amount Mini (Large) Gel	Time (min)	Dark Incubation	Re-use of Solution
1	Fixation	100 mL (250 mL)	2 × 30	Not Required	Yes
2	Washing	100 mL (250 mL)	2 × 15	Not Required	No
3	Staining	65 mL (150 mL)	120	Required	Yes
4	Destaining	100 mL (250 mL)	4 × 30	Required	Yes
5	Washing	100 mL (250 mL)	2 × 5	Required	No

two-dimensional (2-D) gel (*see* **Table 1**). Therefore, this practical change in staining solution alone reduces the overall cost by nine-fold for performing a large-scale phosphoproteomics analysis. Moreover, the required volume of other solutions, such as fixation and destaining, is half of that recommended by manufacturer. The optimized conditions of this modified protocol are summarized for both mini (size 8 cm × 8 cm × 1 mm) and large (size 26 cm × 20 cm × 1 mm) gels in **Table 1**. Application of this simple, economical protocol to large scale identification and quantification of phosphoproteins expressed in developing rapeseed *(8)* has further demonstrated its suitability and reproducibility for conducting large-scale phosphoproteomics.

2. Materials

1. miniVE vertical electrophoresis unit (GE Healthcare, Piscataway, NJ).
2. Ettan DALTtwelve separation unit (GE Healthcare, Piscataway, NJ).
3. Mini-gel staining tray, size 13.75 cm × 13.75 cm × 5.25 cm (GLAD WARE, Oakland, CA) (*see* **Notes 1** and **2**).
4. Large-gel staining tray, size 26 cm × 20 cm × 10 cm (Daigger, Vernon Hills, IL; catalog number EFRCP 124PCLE) (*see* **Notes 1** and **2**).
5. SDS (sodium dodecyl sulfate) loading buffer: 5% (v/v) glycerol, 60 m*M* SDS, 100 m*M* DTT, 0.03 m*M* bromphenol blue, 60 m*M* Tris-HCl, pH 6.8 in deionized water.
6. Orbital shaker (GeneMate, OS350, ISC Bioexpress, Kaysville, UT).
7. Pro-Q DPS (Invitrogen, Carlsbad, CA; product number P33301). Store at 4°C (*see* **Note 3**).
8. PeppermintStick phosphoprotein molecular weight standards (Invitrogen, Carlsbad, CA; product numbers P33350). Store in aliquots at −20°C (*see* **Note 4**).
9. Methanol (Fisher Scientific, Houston, TX).
10. Acetic acid (Fisher Scientific, Houston, TX).
11. Acetonitrile (Fisher Scientific, Houston, TX).
12. Powder-free nitrile gloves (Fisher Scientific, Houston, TX).
13. Fixation solution: 50% methanol (v/v), 10% acetic acid (v/v) in deionized water. (*see* **Note 5**).

14. Washing solution: deionized water (18.2 MΩ conductivity).
15. Staining solution: three-fold diluted Pro-Q DPS (v/v) in deionized water.
16. Destaining solution: 50 m*M* sodium acetate-acetic acid, pH 4.0, 20% acetonitrile (v/v) in deionized water. To prepare one liter of destaining solution, combine 50 mL of 1000 mM sodium acetate, pH 4.0, 750 mL of deionized water, and 200 mL of acetonitrile (*see* **Note 6**).
17. Fluorescence image capture device: FLA 5000 laser scanner (Fuji Medical Systems, Stamford, CT).
18. Image quantification software: Image Gauge Analysis software (Fuji, Stamford, CT).
19. ImageMaster 2D Platinum software version 5 (GE Healthcare, Piscataway, NJ).

3. Methods

Since this chapter is specifically focused on describing the complete protocol of the modified Pro-Q DPS, details on mini- and large-gel preparation and extraction conditions for phosphoprotein analyses are not mentioned here. Any method can be used to make the desired SDS polyacrylamide gel (SDS-PAG) and to separate the proteins. We used the Laemmli ***(9)*** method for the development of this modified Pro-Q DPS protocol. After protein separation, the gel is stained using the modified Pro-Q DPS protocol. **Table 1** summarizes all steps including the required volumes of solutions for mini- and large-size gels, total incubation times and conditions, and whether solutions can be reused. Gels should be imaged immediately after Pro-Q DPS staining.

3.1. One- and Two-Dimensional Gels

1. Prepare 12% SDS-PAG of size 8 cm × 8 cm × 1 mm, termed mini- or 1-D gel (*see* **Note 7**).
2. Prepare 12% SDS-PAG of size 26 cm × 20 cm × 1 mm, termed large- or 2-D gel (*see* **Note 7**).
3. Separate proteins on gel, beside a lane containing PeppermintStick phosphoprotein molecular weight standards, at the desired voltage and time period (*see* **Note 4**).

3.2. Staining the Gel

1. Immerse the gel in fixation solution (100 mL for mini gel and 250 mL for large gel). Incubate at room temperature (RT) with constant shaking on an orbital shaker at a speed of 35 rpm for 30 min. Decant fix solution and repeat (*see* **Notes 2** and **8–12**).
2. Immerse the gel in washing solution (100 mL for mini gel and 250 mL for large gel) for 15 min. Decant wash solution and repeat (*see* **Notes 8, 9,** and **13**).
3. Incubate the gel in staining solution (65 mL for mini gel and 150 mL for large gel) in the dark for 2 h (*see* **Notes 8, 9, 14,** and **15**).
4. Immerse the gel in destaining solution (100 mL for mini gel and 250 mL for large gel) and incubate in the dark for 30 min. Decant destain solution and repeat this step three more times. The total required destaining time is 2 h (*see* **Notes 8, 9, 11,** and **16**).
5. Wash the gel with deionized water (100 mL for mini gel and 250 mL for large gel) in the dark for 5 min. Decant deionized water and repeat (*see* **Notes 8, 9,** and **17**).

3.3. Gel Scanning and Analysis

1. Scan the gel using a laser imager with 532-nm excitation and 580-nm bandpass emission filter (Fujifilm FLA 5000). Collect and analyze data as 100-µm resolution, 16-bit TIFF files (Image Gauge Analysis software, Fuji, Stamford, CT) (*see* **Notes 18** and **19**).
2. Use Image Gauge Analysis software (Fuji, Stamford, CT) and ImageMaster 2D Platinum software version 5 (GE Healthcare, Piscataway, NJ) to display and analyze data. (*see* **Notes 20** and **21**).

4. Notes

1. These trays are reusable and should be thoroughly cleaned with detergent, rinsed with deionized water, and then finally rinsed with 70% (v/v) ethanol prior to use.
2. Select appropriate tray for mini and large gels. For example, we routinely use a tray size of 13.75 cm × 13.75 cm × 5.25 cm and 26 cm × 20 cm × 10 cm for mini (8 cm × 8 cm × 1 mm)- and large (26 cm × 20 cm × 1 mm)-size gels, respectively. Volumes of solutions required to immerse the mini- and large- size gels are provided in **Table 1**.
3. Pro-Q DPS is light-sensitive and should be protected from light at all times. It is stable for six months even if it is stored at RT. In our experience, storage of Pro-Q DPS at 4°C prolongs its stability by at least two-fold. After this storage period, we found that the stability of stain drops sharply resulting in the loss of specificity. Therefore, it is recommended that the Pro-Q DPS activity should be checked on small size gel using PeppermintStick phosphoprotein molecular weight standards (or any protein standard containing ovalbumin and casein) before performing large-scale experiments. Additionally, we recommend all analytical gels should contain a molecular marker containing positive and negative phosphoprotein controls.
4. PeppermintStick phosphoprotein molecular weight standards carry two phosphorylated (ovalbumin and bovine β–casein of 45.0 and 23.6 kDa, respectively) and four non-phosphorylated (β-galactosidase, bovine serum albumin, avidin, and lysozyme of 116.25, 66.2, 18.0, and 14.4 kDa, respectively) standards. Such protein markers are important to include in any Pro-Q DPS-based experiments to exclude false positive identification by normalizing the detected phosphoprotein bands or spots against positive and negative phosphoprotein markers. It should be noted that any protein standard with ovalbumin and/or casein can be used for this purpose.
5. Unless stated otherwise, all solutions should be freshly prepared in deionized water (18.2 MΩ conductivity) before use.
6. It is recommended to prepare a stock solution of 50 m*M* sodium acetate, pH 4.0. Use acetic acid to adjust the pH to 4.0. Store at RT.

7. We used 4% T, 2.6% C stacking gels, pH 6.8, and 12% T, 2.6% C separating gels, pH 8.8. The % T is the total monomer concentration expressed in grams/100 mL, and % C is the percentage of cross-linker. The stacking and separating gel buffer concentrations were 125 m*M* Tris-HCl, pH 6.8, and 375 m*M* Tris-HCl, pH 8.8, respectively. Gels can be stored at 4–8°C for a week if wrapped to prevent dehydration. The reservoir buffer concentration was 25 m*M* Tris, 192 m*M* glycine, pH 8.3. All gel and reservoir buffers carried SDS to a final concentration of 0.1% (w/v). For mini (1-D)-gel electrophoresis, proteins were heat denatured for 5 min at 75°C in SDS loading buffer and cooled to RT before loading to wells. The electrophoresis units used for mini (1-D) and large (2-D) gels were miniVE vertical electrophoresis unit and Ettan DALTtwelve separation unit (GE Healthcare, Piscataway, NJ, USA), respectively.
8. Unless stated otherwise, all steps during this modified Pro-Q DPS procedure should be performed at RT with constant shaking on an orbital shaker at a speed of 35 rpm.
9. To decant the solution, it is important to wear powder-free nitrile gloves. Hold the tray with one hand and use the other to hold the gel in the tray. Tilt the tray to decant most of the solution. Be careful not to press the gel hard, otherwise gel may break or have finger impression.
10. This fixation step is important for fixation of proteins in the gel and at the same time to remove SDS from the gel matrix. It should be noted that the signal intensity and linear dynamic range decrease dramatically if the gel is not incubated with fixation solution for required period of time. We also recommend washing the gel twice with deionized water at RT for 10 min each prior to the fixation step (i.e. **Step 1** of **Subheading 3.2**). This is particularly important for a large-format 2-D gel, as it helps in washing out the SDS from the gel surface and to some extent from the gel matrix.
11. The one-time used fixation solution can be reused at least once more without compromising the signal intensity and linear dynamic range. Agrawal and Thelen *(7)* defined the reuse of solution "as a solution that has been used one-time and reserved for subsequent experiments."
12. If one wants to process the gel the next day, the fixation step is a good step to **stop** the protocol. In this situation, the gel can be left in the fresh fix solution overnight with constant shaking. However, we recommend completing the entire staining procedure including gel imaging the same day. This is because, we have experienced that overnight fixation of the gel requires longer washing time (may need additional wash) than mentioned in the **Table 1** (*see* **Step 2** of **Subheading 3.2**). Moreover, proper care is necessary to prevent the gel from folding-over after adding the washing solution. If this becomes problematic, one way to prevent gel

folding is to increase the volume of washing solution and the shaking speed. We found that gel folding causes uneven removal of methanol and acetic acid from the gel matrix (*see* **Note 13**) and as a consequence uneven detection of phosphoproteins having varying degree of signal intensity with Pro-Q DPS.

13. Removal of all methanol and acetic acid from the gel is critical. Residual methanol or acetic acid appears to quench Pro-Q DPS signal and reduce sensitivity ***(5, 6; Agrawal and Thelen, unpublished data)***. Residual methanol or acetic acid can also cause higher gel background (***Agrawal and Thelen, unpublished data***).
14. To create dark conditions for staining, we have used cardboard boxes (light-proof) or black plastic garbage bags to completely cover the gel tray. Minimize exposure to light during solution changes.
15. Staining solution can be reused only for qualitative detection of phosphoproteins. Linear quantitative range of Pro-Q DPS is lower with reused stain ***(7)***.
16. The destaining step reduces the gel background and also the non-specific signal. It should be noted that gels thicker than 1 mm may take longer than the recommended 2 h destaining time.
17. Keep the gel in deionized water at RT after washing step until imaging of the gel is finished.
18. We use an FLA 5000 laser scanner as fluorescence image capture device for imaging Pro-Q DPS stained gels. However, any flatbed fluorescent imager can be used for image capture. Pro-Q DPS has 555/580 nm excitation/emission maxima. Therefore, stained gels can be best imaged using excitation lasers or LEDs with a range of 532–560 nm coupled with a 580 nm longpass or a 600 nm bandpass emission filter.
19. After gel imaging, the gel can be directly stained with a total-protein stain, such as colloidal Coomassie Brilliant Blue, silver stain, or SYPRO Ruby protein gel stain. This should be performed immediately after gel imaging. The total-protein stain provides a landmark for excising the phosphoproteins for their identification by mass spectrometry and helps in determining the relative phosphorylation state of a given protein.
20. With these software programs, fluorescent protein signals in 1-D gel are displayed as dark bands or in 2-D gel dark spots.
21. Any 1-D or 2-D gel image analysis software can be used for band and spot quantification, respectively.

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Electroelution of Proteins from Polyacrylamide Gels

Paul Jenö and Martin Horst

1. Introduction

Understanding the function of proteins requires determination of their structures. Advances in chemical technology make it possible to obtain a picture of global protein expression in model organisms such as the yeast *Saccharomyces cerevisiae*. Instrumental to these breakthroughs was the development of high-resolution two-dimensional gel electrophoresis and mass spectrometric means for rapid detection and analysis of peptides and proteins. While sequence information on single spots separated by two-dimensional electrophoresis can be quickly obtained by today's technology, structural characterization of proteins expressed in low abundance is still a difficult task. For example, in our attempts to isolate components of the import machinery of yeast mitochondria, some proteins turned out to be expressed at extremely low levels, and it soon became evident that conventional purification techniques were impractical to obtain these proteins in amounts sufficient for structural analysis. Furthermore, some proteins of the mitochondrial import machinery were rapidly degraded by proteases, and they could be obtained intact only by first denaturing mitochondria with trichloro-acetic acid and then solubilizing them in boiling sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ***(1)***. This in turn made the separation of several milligrams of denatured proteins necessary, which was best achieved by preparative SDS-PAGE followed by electroelution of individual proteins.

Proteins isolated by electroelution usually contain large amounts of salts and SDS, which interfere with enzymatic digestion or Amino-D (N)-terminal sequencing. A number of methods are available to remove SDS from proteins, including precipitation of the detergent by organic solvents ***(2,3)***, and solvent extraction with ***(4)*** or without ion-pairing reagents ***(5)***. However, they all

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suffer from the disadvantage that once the detergent is removed, many proteins become virtually insoluble in buffers lacking SDS. Alternatively, chromatographic methods can be used to remove SDS from proteins. Simpson et al. ***(6)*** described desalting of electroeluates based on the finding that certain reverse-phase matrices retain proteins at high organic modifier concentrations, whereas small molecular weight compounds are not. This allows bulk separation of salts, SDS, and Coomassie Blue staining components from protein material. Based on a different stationary phase, a similar approach was developed ***(7)*** to separate protein from contaminants originating from the electroelution process. In this method, electroeluted proteins are applied to a poly (2-hydroxyethyl) aspartamide-coated silica, which provides a polar medium for binding of proteins when equilibrated at high organic solvent concentration ***(8)***. Bound proteins are then eluted with a decreasing gradient of organic modifier, allowing recovery of protein free of SDS and buffer salts. Although the methodology is similar to the one described by Simpson et al. ***(6)***, proteins tend to adsorb to the poly (hydroxyethyl) aspartamide matrix at lower *n*-propanol concentrations than to reverse-phase matrices, therefore minimizing the danger of irreversible protein precipitation on the stationary phase.

In this chapter, we describe an electroelution procedure that has worked well with proteins in the molecular weight range of 20–100 kDa isolated from yeast mitochondrial membranes. In addition, a method is described which allows the desalting of these proteins into volatile buffer systems by hydrophilic-interaction chromatography.

2. Materials

1. Electrophoresis apparatus: Preparative electrophoresis is carried out on 16 × 10 cm separating and 16 × 2 cm stacking gels of 1.5 mm thickness with the buffer system described by Laemmli ***(9)***. For sample application, a preparative sample comb of 2 cm depth and 14 cm width is used. Up to 2.5 mg total protein is applied onto one preparative slab gel. Samples are electrophoresed at 15 V for 14 h. Chemicals used for electrophoresis (acrylamide, *N*,*N* -methylene *bis*-acrylamide, ammonium persulfate, *N*,*N*,*N′*,*N′* -tetramethyl-enediamine, SDS, and Coomassie Blue R250) are electrophoresis-grade and are purchased from Bio-Rad (Hercules, CA). Methanol and acetic acid used for staining are *pro analysis* (p.a.) grade from Merck (Darmstadt, Germany). Unless stated, all other chemicals used are of the highest grade available.
2. Staining solution: 0.125% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) methanol, 10% acetic acid. Filter the staining solution over 320-µm filters (Schleicher and Schuell, Dassel, Germany) before use.
3. Destaining solution: 50% methanol, 10% acetic acid.
4. Electroelution apparatus: BIOTRAP from Schleicher and Schuell (Dassel, Germany, “Elutrap” trademark in the US and Canada).
5. BT1 and BT2 membranes for electroelution (Schleicher and Schuell).

6. Electroelution buffer: 25 m*M* Tris, 192 m*M* glycine, 0.1% SDS. This buffer is prepared with NANOpure water from a water purification system (Barnstead, Dubuque, IA, USA) and electrophoresis-grade SDS (Bio-Rad).
7. Electrodialysis buffer: 15 m*M* NH_4HCO_3, 0.025% SDS. Prepare the buffer with NANOpure water and electrophoresis-grade SDS (Bio-Rad).
8. High-pressure liquid chromatography (HPLC) equipment: we are using a Hewlett Packard HP1090M (Palo Alto, CA) liquid chromatograph connected to a diode array UV detector (Hewlett Packard, Palo Alto, CA) for operating columns of 4.6 × 200 mm or 2.1 mm diameter.
9. Poly (hydroxyethyl) aspartamide (PHA) columns: 5 µm particle size, 20 nm pore size, 4. × 200 mm or 2.1 × 200 mm (PolyLC, Columbia, MD).
10. Solvents for hydrophilic interaction chromatography: solvent A: 100% *n*-propanol (HPLC grade, Merck), 50 m*M* formic acid (Merck, analytical grade); solvent B: 50 m*M* formic acid in water (NANOpure).

3. Method

1. After electrophoresis, stain the gel for 15 min with Coomassie Blue. To prevent irreversible fixation of the protein, destaining is observed on a light box. As soon as the protein of interest becomes visible, the band is cut out with a razor blade. The gel piece is washed once with 10-mL of 1 *M* Tris-HCl, pH 8.0 for 5 min, followed by three 10-mL washes with water for 5 min each. Cut the gel piece with a razor blade into small cubes. Equilibrate them in 10-mL of electroelution buffer for 10 min with occasional shaking. In the meantime, assemble the electroelution apparatus.
2. The electroelution device is a block of polycarbonate (160 × 30 × 30 mm) that has an open channel along its axis (**Fig. 1**). An elution chamber that holds the polyacrylamide pieces is formed with trap inserts between points C and F of the body. During the elution process, the protein is trapped into a chamber formed between points F and G (**Fig. 1**). The device works with two types of membranes having different ion permeabilities: the BT1 membrane retains all charged macromolecules larger than 5 kDa, whereas buffer ions can freely permeate under the influence of an electric field. The macroporous BT2 membrane acts as a barrier that prevents particulate matter from entering the trap. It also keeps the buffer from flowing into the trap when the electric field is switched off, preventing dilution of the protein in the trap.

   Slide BT1 membranes between the clamping plates and the trap inserts at positions A and G of the BIOTRAP apparatus (**Fig. 1**). Since the BT1 membrane is an asymmetric membrane with two different surfaces, make sure that they are mounted in the proper orientation. The BT1 membrane is delivered moist and should not dry out. Buffer should be added within 5 min after insertion of the membranes. Insert a BT2 membrane at points C and F. If necessary, smaller elution chambers can be formed by inserting the BT2 membrane at positions D or E. Tighten the pressure screws to hold the membranes in place. Transfer the gel pieces with a spatula into the elution chamber formed between the two BT2 membranes inserted at positions C and F. Carefully overlay the gel pieces with electroelution buffer until the

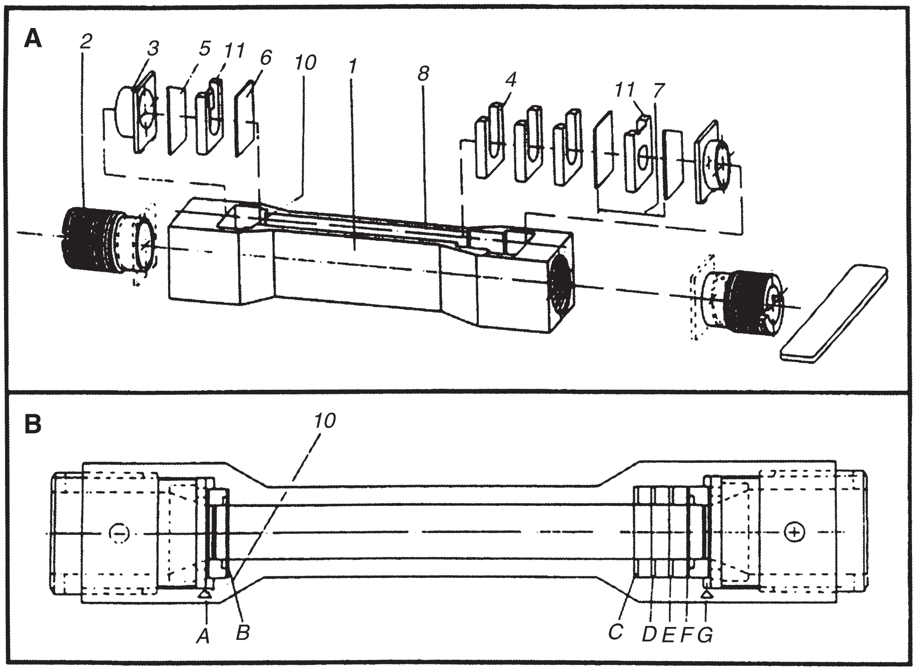

Fig. 1. Side **(A)** and top **(B)** view of the electroelution device. The apparatus is assembled by inserting a BT1 membrane at points A and G. The trap that collects the protein during the elution process is formed between points F and G. The chamber that holds the gel pieces is formed by inserting a BT2 membrane at point C. Smaller elution chambers can be made by inserting the BT2 at point D or E. The trap inserts and membranes are fixed by clamping plates, which press the trap inserts against the cell body. *1*, Cell body; *2*, pressure screw; *3*, clamping plate; *4*, trap inserts; *5*, membrane BT1; *6*, membrane BT2; *7*, trap chamber; *8*, elution chamber; *10*, mark for correct orientation of membrane BT1; *11*, trap insert for membranes BT1 (modified with permission from Schleicher and Schuell).

level of the liquid is approx 5 mm above the gel pieces. After some minutes, the trap is filled with buffer by seeping through membrane BT2. Make sure enough liquid is in the elution chamber so that the gel pieces remain completely immersed. Place the electroelution device into a horizontal electrophoresis chamber with the + mark directed toward the anode of the electrophoresis chamber. The dimensions of the horizontal electrophoresis tank are as follows: 30 cm length, 20 cm width, and 7 cm depth. The T-shaped table for agarose gels is 3 cm from the bottom. Add enough electroelution buffer to the electrophoresis chamber to fill half of the BIOTRAP (approx 3 L). Electroelute the protein for 18 h at 100 V (the current will be in the range of 70–90 mA). The volume into which the eluted protein is recovered depends on the buffer level inside the BIOTRAP and ranges from 200–800 µL.

3. Replace the electroelution buffer with 3 L of electrodialysis buffer and electrodialyze the sample for 6 h at 40 V against 15 m*M* NH_4HCO_3, 0.025% SDS.
4. Remove the eluted protein from the trap. Be careful not to perforate the BT1 membrane with the pipet tip! Rinse the trap twice with 100 µL of fresh electrodialysis buffer. Combine the dialysate and the washes. The solution is dried in a Speed Vac concentrator and stored at –20°C.
5. For desalting of the electroeluted protein, equilibrate the PHA column with solvent A (65% *n*-propanol, 50 m*M* formic acid). Electroeluates containing > 5 µg of protein are desalted on 4.6 mm internal diameter columns, which are operated at a flow rate of 0.5 mL/min. Less than 5 µg protein are chromatographed on 2.1 mm internal diameter columns at 75 µL/min. The effluent is monitored at 280 nm.
6. Dissolve the dried protein in a small volume of water (50–100 µL). The dried SDS efficiently solubilizes the protein. *n*-propanol is added to 65% final concentration. The sample is then applied in 50-µL aliquots onto the PHA column. After each injection, a number of UV-absorbing peaks, caused by Coomassie blue components, elute from the column. It is important that these components are completely washed out before the next aliquot is injected. With this procedure, the protein is efficiently concentrated on the column inlet. After the entire sample has been applied, the gradient is initiated, which is developed in 10 min from 65% *n*-propanol/50 m*M* formic acid to 50 m*M* formic acid. The protein elutes at the end of the gradient and is now devoid of any salt or SDS (**Fig. 2**).

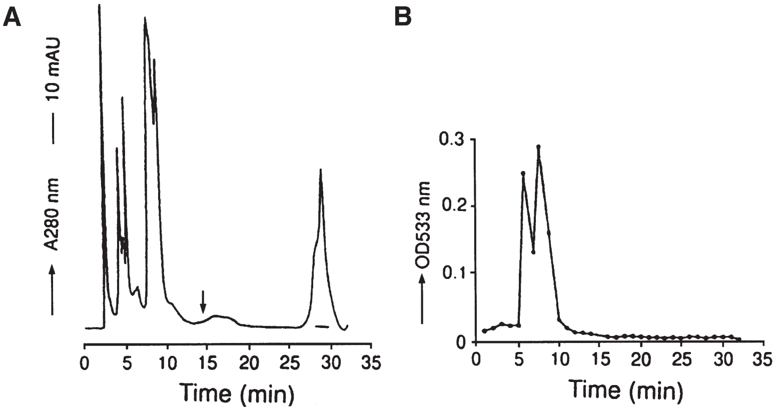

Fig. 2. Removal of SDS from an electroeluate. **(A)** 20 µg of a 45-kDa mitochondrial outer membrane protein in 50 µL was injected onto a PHA column (4.6 × 200 mm) that had been equilibrated in 70% *n*-propanol–50 m*M* formic acid. After the baseline had stabilized, the gradient was initiated (marked with an arrow). Bound protein was eluted with a linear 10-min gradient from 70% *n*-propanol–50 m*M* formic acid to 50 m*M* formic acid at a flow rate of 0.5 mL/min. The protein eluted between 27 and 30 min. **(B)** Fractions of 500 µL were collected and tested with Fuchsin red for the presence of SDS. (Modified with permission from **ref.** *7*).

7. The protein is now ready for further protein structural characterization. It can be directly subjected to automated Edman degradation. For enzymatic fragmentation, residual *n*-propanol has to be removed in the Speed Vac prior to adding the protease.

4. Notes

1. To locate the protein of interest in the gel, a staining method has to be chosen so that maximal sensitivity with minimal fixation is obtained. A number of methods exist to visualize proteins in the polyacrylamide matrix, such as formation of insoluble protein–SDS complexes with potassium ***(10)***, or precipitation of SDS by 4 *M* sodium acetate ***(11)***. We found staining of complex protein patterns with these methods difficult, as they tend to produce diffuse bands. Staining the gel for 15 min with Coomassie Blue is sufficient to visualize also faint bands without fixing the protein irreversibly. To minimize fixation of proteins, destaining is carried out on a light box, so that the band of interest can be sliced out of the gel as soon as it becomes visible.
2. The electroelution apparatus routinely used in our laboratory was originally described by Jacobs and Clad ***(12)*** and is commercially available from Schleicher and Schuell. We found this type of apparatus very reliable for routine use. The volume of the elution chamber can be adjusted depending on the volume of the gel pieces used. The volume can be increased or decreased by varying the position of the BT2 membranes between positions C and F (*see* **Fig. 1**). By forming the smallest possible elution chamber, one can process Coomassie Blue-stained bands from a single one-dimensional analytical PAGE. With the larger elution chamber, up to five preparative gels can be processed at a time. However, other suitably constructed devices will give identical results.
3. So far we have eluted proteins between 20 and 100 kDa with protein amounts ranging from 10 to 50 µg per band. After elution and dialysis, proteins are typically recovered in volumes of between 300 and 800 µL. However, elution of <10 µg of protein becomes difficult owing to the large volume of the trap, nonspecific adsorption of proteins to the BT1 membrane, or microleaks in the trap. In such cases, we prefer to run several preparative gels in parallel and pool multiple protein slices until the required amount of protein for electroelution is obtained. For low amounts of protein, several commercially available devices can be used which elute proteins into smaller volumes than the BIOTRAP apparatus.
4. Electroelution of proteins into ammonium hydrogen carbonate would be preferable, since NH_4HCO_3 can be removed by lyophilization. However, owing to the low buffering capacity of NH_4HCO_3, the pH of the buffer

drops after 4 h, rendering electroelution of high molecular weight proteins difficult due to their slow elution from the gel pieces. Therefore, it is preferable to electroelute proteins into Tris–glycine–SDS followed by electrodialysis into NH_4HCO_3-containing buffers.

5. When using 0.1% SDS in the electroelution buffer, the micelles formed in front of the BT1 membrane lead to massive accumulation of SDS in the trap. The detergent and low molecular weight contaminants can be easily removed by hydrophilic interaction chromatography with simultaneous desalting of the protein into a volatile buffer ***(7)***. Alternatively, the procedure devised by Simpson et al. ***(6)*** can be used to desalt the electroeluted protein.
6. Hydrophilic interaction chromatography of proteins requires careful control of the solvent composition. To test column performance, we use a test mixture consisting of cytochrome *c*, ovalbumin, and bovine serum albumin. Use of this test mixture allows finding the minimal *n*-propanol concentration at which the electroeluted protein binds to the stationary phase without the risk of precipitation by the organic solvent. Protein binding usually occurs at *n*-propanol concentrations between 60% and 65%. When performing electroelution with Tris–glycine buffers, direct application of the electroeluate is not possible due to precipitation of buffer salts above 50% *n*-propanol concentration. In such cases, buffer exchange into 0.1 *M* NH_4HCO_3, 0.01% SDS is carried out by electrodialysis in the electroelution apparatus. Ammonium bicarbonate can be removed by Speed Vac drying. The residual SDS facilitates solubilization of the dried protein with water. No salt precipitation is observed when adding *n*-propanol to 65% final concentration

   The solvent system used to elute bound proteins contains 50 m*M* formic acid. Unfortunately, this precludes detection of proteins at wavelengths below 250 nm. Since most proteins contain tyrosine and tryptophane residues, detection of eluting proteins is done at 280 nm. For proteins lacking tyrosines or tryptophanes, 0.05% trifluoroacetic acid (TFA) can be used instead of formic acid. However, exposure of the stationary phase to TFA should be kept to an absolute minimum, since TFA greatly reduces the lifetime of the column.

   Efficient removal of small molecular weight contaminants such as SDS and Coomassie Blue requires small injection volumes of the electroeluted protein. When using 4.6 mm i.d. columns, 50 µL injections were found to be optimal; for 2.1 mm i.d. columns, the injection volume is reduced to 20 µL. Larger volumes are concentrated on the column inlet with multiple injections. In such cases, the column should be allowed to reequilibrate between individual injections, otherwise loss of protein in the breakthrough volume occurs.

   Electroeluted proteins chromatographed by hydrophilic interaction often display unsymmetrical peaks. This may indicate the presence of several different proteins in the eluate, which are partially resolved by the stationary phase, or heterogeneity of a single protein, which became modified during electrophoresis. Since the main purpose is to free the electroeluted protein from SDS and Coomassie Blue, we tend to elute the protein into one single peak by running very steep gradients, rather than trying to separate the eluting material into single components. This can be subsequently achieved by two-dimensional gel electrophoresis, or by reverse-phase chromatography after removal of residual *n*-propanol.

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Autoradiography and Fluorography of Acrylamide Gels

Antonella Circolo and Sunita Gulati

1. Introduction

Autoradiography detects the distribution of radioactivity on gels or filters by producing permanent images on photographic film. It is frequently used in a variety of experimental techniques ranging from Southern and Northern blot analysis ***(1)***, to visualization of radioactive proteins separated in a sodium dodecyl sulfate (SDS)-Polyacrylamide gels ***(2)***, to detection of nuclear factors bound to a labeled DNA probe in gel shift analysis ***(3)***, and to localization of DNA bands in sequencing gels ***(4)***.

Autoradiographic images are formed when particles emitted by radioactive isotopes encounter the emulsion of an X-ray film and cause emission of electrons from silver halide crystals that, in turn, react with positively charged silver ions, resulting in the precipitation of silver atoms and the formation of an image ***(5)***.

^{35}S and ^{32}P isotopes are the most commonly used isotopes for autoradiography. ^{35}S is a β-emitter of relatively low energy (0.167 MeV). Thus ^{35}S particles penetrate a film to a depth of 0.22 mm, generally sufficient to interact with the emulsion in the film, as long as care is taken to assure that the film and the source of radioactivity are in direct contact, and that no barriers are posed between the film and the gel. In addition, gels must be completely dry before autoradiography. ^{32}P is a β-emitter with an energy of 1.71 MeV. Therefore, its particles penetrate water or other materials to a depth of 6 mm, passing completely through a film. In this case, gels or filters do not need to be dry, since water will not block particles of this energy and may be covered with a clear plastic wrap before autoradiography. The efficiency of ^{32}P-emitted β-particles is enhanced when an intensifying screen is placed behind the X-ray film, because radioactive particles that pass through the film cause the screen to emit photons that

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sensitize the film emulsion. The use of intensifying screens results in a fivefold increased enhancement of the autoradiographic image when the exposure is performed at low temperature (–70°C). In general, calcium tungstate screens are the most suitable because they emit blue light to which X-ray films are very sensitive ***(6)***.

Radiation of sufficiently high energy (e.g., ^{32}P and ^{35}S) can be detected by simple autoradiography, but low energy emissions may not penetrate the coating of the film, and the most sensitive fluorography procedure is used in these cases. In fluorography, the use of fluorescent chemicals increases about 10-fold the sensitivity of detection of weak β-emitters (^{35}S and ^{14}C), and permits detection of radioactivity from ^{3}H, virtually undetectable with simple autoradiography. Gels are impregnated with scintillant or fluors, which come in direct contact with the low-energy particles emitted by ^{35}S, ^{14}C, and ^{3}H. In response to radiation, fluors emit photons that react with the silver halide crystals in the emulsion of the film. Because the wavelength of the emitted photons depends on the fluorescent chemical and not on the radioactive emission of the isotope, the same type of film can be used to detect radioactivity from isotopes of varying energies ***(7)***.

In this chapter, we describe the most common techniques of autoradiography and fluorography, and a double silver-staining method for acrylamide gels that approaches the sensitivity of autoradiography and that may be used for those experiments in which radiolabeling of proteins is not easily obtained (*see* **Note 1**). Autoradiography of wet and dry gels is described in **Subheading 3.1.**, and protocols for fluorography and methods for quantification of radioactive proteins in polyacrylamide gels are given in **Subheading 3.2.** Double silver staining is described in **Subheading 3.3.**

2. Materials

2.1. Autoradiography

1. X-ray film (Kodak XR [Rochester, NY], Fuji RX [Pittsburgh, PA], Amersham [Arlington Heights, IL], or equivalent).
2. Plastic wrap (e.g., Saran Wrap).
3. 3 MM Whatman paper.
4. SDS-PAGE fixing solution: 46% (v/v) methanol, 46% (v/v) water, 8% (v/v) acetic acid glacial. Store in an air-tight container. It is stable for months at room temperature.

2.2. Fluorography

1. X-ray blue-sensitive film (Kodak XAR-5, Amersham, or equivalent).
2. Plastic wrap.
3. 3 MM Whatman paper.
4. SDS-PAGE fixing solution (as in **Subheading 2.1.**).

5. Coomassie blue staining solution: 0.125% Coomassie brilliant blue R250 in SDS-PAGE fixing solution. Stir overnight to dissolve, filter through a Whatman paper, and store at room temperature protected from light. It is stable for several weeks. For longer storage, dissolve the Coomassie blue in 46% water and 46% methanol only, and add 8% acetic acid glacial just before use.
6. Commercially available autoradiography enhancers (acidic acid-based: En^{3}Hance from Dupont [Mount Prospect, IL], water-soluble: Fluoro-Hance from RPI [Dupont NEN, Boston, MA], or equivalent).
7. Hydrogen peroxide (15% solution).
8. Scintillation fluid.

2.3. Double Silver Staining

1. Silver stain kit (Bio-Rad, Hercules, CA).
2. Methanol, ethanol, acetic acid glacial.
3. Solution A: Sodium thiosulfate 436 g/L.
4. Solution B: Sodium chloride 37 g, cupric sulfate 37 g, ammonium hydroxide 850 mL to 1 L of dd H_2O. Store these solutions at room temperature.

3. Methods

3.1. Autoradiography

3.1.1. Wet Gels

When radioactive proteins have to be recovered from the gel, the gel should not be fixed and dried before autoradiography. However, only high-energy isotopes (^{32}P or ^{125}I) should be used.

1. At the end of the electrophoresis, turn off the power supply and disassemble the gel apparatus. With a plastic spatula, pry apart the glass plates, cut one corner of the gel for orientation, carefully remove the gel, place it over two pieces of Whatman paper, and cover with a plastic wrap, avoiding the formation of bubbles or folds.
2. In a dark room, place the gel in direct contact with the X-ray film, and place an intensifying screen over the film. Expose for the appropriate length of time at room temperature or freeze at −70°C.
3. Develop the film using an automatic X-ray processor or a commercially available developer as following: immerse the gel for 5 min in the X-ray developer, rinse in water for 30 s, transfer into the fixer for 5 min, rinse in running water for 10–15 min, and let dry. All the solutions should be at 18–20°C.

If the exposure of the gel is made at −70°C, allow the cassette to warm to room temperature and wipe off any condensation before opening. Alternatively, develop the film immediately as soon as the cassette is removed from the freezer, before condensation forms. If an additional exposure is needed, allow the cassette to dry completely before reusing.

Cardboard exposure holders may work better with wet gel, since metal cassettes may compress the gel too tightly. When an intensifying screen is used, the film should be exposed at −70°C. The screen enhances the detection of radioactivity up to 10-fold, but may decrease the resolution.

3.1.2. Dry Gels

Gels containing urea should always be fixed to remove urea crystals. When high resolution is required, gels should always be dried before exposure to film. Gels should also be dried when ^{35}S or other low-energy β-emitters are used as radioactive tracers. For improved sensitivity and resolution, gels containing ^{32}P should also be fixed and dried before autoradiography.

1. At the end of the electrophoresis, turn off the power supply, disassemble the gel apparatus, and carefully pry open the two glass plates with a plastic spatula. (To assure that the gel will adhere to one glass plate only, one of the glass plates should be treated with silicon before casting the gel.)
2. Cut one corner of the gel for orientation, place the gel with the supporting glass plate into a shallow tray containing a volume of fixing solution sufficient to cover the gels, and fix for 30 min (the time necessary for fixation varies according to the thickness of the gel, but longer times do not have deleterious effects).
3. After fixation, carefully remove the plate from the tray, taking care not to float away the gel, or with a pipet connected to a vacuum pump, remove the solution from the tray and carefully from the glass plate. Place a wet piece of Whatman paper over the gel, being careful to avoid formation of bubbles and folding of the gel. Blot dry the Whatman paper with dry paper towels, applying gentle pressure.
4. Flip over the plate and maintain it exactly over the tray of a gel dryer. Carefully begin to detach the gel from one corner of the glass plate. The gel will remain adherent to the Whatman paper and will detach easily from the plate. Carefully cover the gel with plastic wrap, avoiding folding, and then dry the gel in a slab gel vacuum dryer for 1 h at 80°C (*see* **Notes 2–6**).
5. When the gel is dry, remove the plastic wrap and expose to X-ray film. After an appropriate length of time, develop the film as described in **Subheading 3.1.1., step 3**.

To increase the resolution of the radiogaphy, gels in which several bands of similar molecular mass are visualized, should be exposed at room temperature, without an intensifying screen. The use of a screen will result in increased intensity of the radioactive signal, but in decreased sharpness of the image. SDS-PAGE or other gels in which fewer bands need to be resolved may be exposed with an intensifying screen.

3.2. Fluorography

Gels containing weak β-emitters (^{35}S, ^{14}C, and ^{3}H) should be fixed, impregnated with autoradiography enhancers, and dried to reduce the film exposure time necessary for visualization of radioactive bands. SDS-PAGE should also be stained if nonradio-labeled mol-wt markers are used and for quantitative experiments (e.g., immunoprecipitation, detection of cell-free translated products, and so forth) where the radioactivity contained in specific proteins is to be measured in bands cut out from the gel ***(8,9)***.

1. Turn off the power supply, remove the gel from the mold, cut a corner for orientation, and place the gel on a tray containing Coomassie blue-staining solution (the volume of the solution should always be adequate to cover the gel, so that it can float freely). Incubate for 45 min at room temperature with gentle shaking.
2. Remove the staining solution, and replace with SDS-PAGE fixing solution (the staining solution can be filtered through filter paper and reused as long as the radioactivity on it remains low, or until the color changes from blue to purple).
3. Incubate overnight at room temperature with gentle shaking, and replace the fixing solution at least once to accelerate the destaining. Gels should be destained until the mol-wt markers are clearly visible and the background is clear (*see* **Note 7**).
4. Discard the fixing solution in accordance with radioactive liquid waste disposal procedures, and add the autoradiography enhancer.
5. If the enhancer used is based on acetic acid (e.g., En^{3}Hance from Dupont or its equivalent), the gel can be soaked in the enhancer without rinsing, and **steps 6–8** should be followed (*see* **Note 2**).
6. Allow the gel to impregnate with enhancer for 1 h with gentle shaking. Initially, a white precipitate may form on the surface of the gel, but it will disappear within the first 15 min of impregnation. Following impregnation, discard the used enhancer solution (do not mix with waste containing NaOH, $NaHCO_3$, and so forth). Add cold tap water to the gel to precipitate the fluorescent material and incubate the gel in water for 30 min. At this stage, the gel should appear uniformly opaque.
7. After the precipitation step, carefully place the gel over two pieces of wet filter paper, cover with plastic wrap, and dry under heat (60–70°C) and vacuum for 1–2 h on a slab gel dryer.
8. Remove the plastic wrap, tape the gel on a rigid support, and place it against a suitable blue-sensitive X-ray film, with an intensifying screen. Expose at −70°C for an appropriate length of time. Do not store the gel at room temperature for >48 h before exposure, since evaporation of the fluors may occur, resulting in reduced sensitivity. For a longer period of storage prior to exposure, freeze the gel at −70°C.
9. If water-soluble fluorography solutions are used (e.g., Fluoro-Hance from R.P.I. or equivalent), the gel must be equilibrated in water after destaining, and steps 10 and 11 should be followed.
10. Discard the destaining solution, and wash the gel in distilled water for 30 min at room temperature, with shaking.
11. Discard the water and impregnate the gel with the enhancer for 30 min at room temperature with shaking. Remove the enhancer, place the gel over two wet pieces of filter paper, cover with a plastic wrap, and dry under vacuum with heat (60–80°C)

for 2 h (*see* **Note 3**). Expose the gel as described above. (Flouro-Hance can be reused, but should be discarded as soon as the solution shows sign of discoloration) (*see* **Notes 2–7**).

If the gels are not stained with Coomassie blue, after electrophoresis, place the gel in SDS-PAGE fixing solution, and incubate for 45 min at room temperature, with gentle shaking. After incubation, discard the fixing solution and impregnate the gel with enhancer as described (**steps 5** or **9**).

12. Radioactivity incorporated into specific proteins can be determined by cutting out the radioactive bands from the dried fluorographed gel.
13. Precisely position the film over the gel. With a sharp blade or scalpel, cut out the area of the gel corresponding to the band on the film. Also cut out an area of the gel free from radioactivity immediately below (or above) the radioactive band, for subtraction of background.
14. Place each gel slice into a scintillation vial (detaching the filter paper from the slice is not necessary), add 1 mL of a 15% solution of hydrogen peroxide, and incubate overnight in a water bath at 60°C to digest the gel and release the radioactivity.
15. After incubation, allow the vials to cool down to room temperature, add scintillation fluid, and measure the radioactivity in a scintillation counter. Alternatively, autoradiographic images can be quantitated by densitometric scanning of different exposures of the film (*see* **Note 8**).

3.3. Double Silver Staining (see Note 9)

1. After electrophoresis, transfer the gel in a glass tray containing 40% methanol and 10% acetic acid, and incubate at room temperature for at least 30 min (longer periods of time have no detrimental effect).
2. Discard this solution, and incubate for 15 min in 10% ethanol, 5% acetic acid.
3. Repeat **step 2** one more time.
4. Add Oxidizer (diluted according to the manufacturer's protocol) and incubate 5 min, taking care that the gel is completely submerged in the solution.
5. Rinse the gel twice in distilled water.
6. Incubate 15 min in double-distilled water.
7. Repeat step 6 until the yellow color is completely removed from the gel.
8. Add Silver reagent (diluted according to the manufacturer's protocol), and incubate for 15 min.
9. Wash the gel once in double-distilled water.
10. Add developer (prepared according to the manufacturer's protocol), swirl the gel for 30 s, discard the solution, and wash once in double-distilled water.
11. Repeat **step 10** and develop the gel until bands appear and the mol-wt markers become clearly visible.
12. Stop the reaction with 5% acetic acid, incubate 5 min, and wash with double-distilled water.
13. Add 3.5% solution A and 3.5% solution B, and incubate 5–10 min, or until the gel is clear.
14. Incubate two times in 10% acetic acid, 30 min each time.

15. Restain the gel by repeating **steps 1–12**.
16. Dry the gel in a slab dryer with heat and vacuum, or use a gel rap (Bio-Rad or equivalent), and dry overnight.

4. Notes

1. Autoradiography of SDS-PAGE is a powerful technique that permits detection of very low amounts of protein. However, in some instances, radioactive protein labeling cannot be easily accomplished. For example, metabolic labeling requires active protein synthesis ***(12)***; thus, proteins present in body fluids or in tissue biopsy cannot be labeled ***(13)***. Moreover, in in vivo animal experiments, it is often difficult to obtain radiolabeled proteins with high specific activity. In this case, silver staining of gels can be used as an alternative method, since it approaches the sensitivity of autoradiography ***(14)***. We have developed a double silver-staining technique that is about 10-fold more sensitive than the conventional silver staining. This method has not been previously published in detail, except for figures presented elsewhere ***(15)*** (*see* **Subheading 3.3.**).
2. Gels of high polyacrylamide concentration (>10%) or gradient gels (acrylamide concentration 5–15%) may crack when dried. This problem is reduced by adding glycerol (1–5%) before drying. When acid-based fluorography enhancers are used, the glycerol should be added during the fluors precipitation step in water after removal of the enhancer. When water-soluble enhancers are used, the glycerol is added during equilibration of the gel in water, before addition of the enhancer. If the concentration of glycerol is too high, gels are difficult to dry, and they may stick to the film. Addition of the enhancer does not increase the cracking. We currently use water-based enhancers for our experiments, because they give sharp autoradiography images and good sensitivity, and can be reused for several gels. Fluorography with commercially available enhancers is simpler and less tedious than the traditional method with PPO-DMSO, and the results are as good as, or better than, those obtained with this method.
3. Enhancers are also used to increase the sensitivity of the autoradiograpy of DNA and RNA of agarose, acrylamide, or mixed gels. When enhancers are used, the gels must be dried at the suggested temperature, because excessive heat will cause damage of the fluors crystal of the enhancer and formation of brown spots on the surface of the gel.
4. Cracking may also be owing to the formation of air bubbles between the gel and the rubber cover of the dryer. This is generally caused by a weak vacuum that is insufficient to maintain the gel well adherent to the paper filter and to the dryer's tray. Air bubbles can be eliminated by rolling a pipet over the rubber cover while the vacuum is being applied.

5. Also, excessive stretching of the gel during the transfer to the filter paper may contribute to cracking, particularly for gels that contain high acrylamide concentration. A filter paper should be placed under the gel when removing it from the solution, and the method described in **Subheading 3.1.2.** should be used for larger gels.
6. It is always necessary to cover the gel with a plastic wrap to prevent sticking to the cover of the dryer. In addition, the vacuum should never be released during the drying procedure until the gel is completely dry, since this will cause the gel to shatter.
7. Staining of the gels with Coomassie blue before fluorography may quench the effect of the enhancer, particularly when low amounts of radioactivity are used. Therefore, the gels must be destained thoroughly, until the background is clear and only the protein bands are stained.
8. X-ray films that are not pre-exposed to light respond to radiation in a sigmoidal fashion, because the halide crystals of the emulsion are not fully activated ***(10)***. In contrast, in a pre-exposed film, the response becomes linear and proportional to the amount of radioactivity, therefore, allowing precise quantitative measurement of radioactivity by scanning the autoradiography ***(11)***. In addition, pre-exposure (preflashing) of the film results in a two-to threefold increase in sensitivity, for levels of radioactivity near the minimum level of detectability ***(7)***, enabling autoradiography of gels containing ^{14}C and ^{3}H radioisotopes to be performed at room temperature, instead of −70°C. To pre-expose a film, a stroboscope or a flash of light (<1 ms) from an electronic flash unit can be used, but it is necessary to reduce the light emission with a Deep Orange Kodak Wratten No. 22, or an Orange Kodak Wratten No. 21 filter, and to diffuse the image of the bulb with two pieces of Whatman No. 1 filter paper placed over the light-emitting lens. The distance between the film and the light source should be determined empirically ***(11)***.
9. In silver staining, surface artifacts can be caused by pressure, fingerprints, and surface drying. Gloves should always be worn when handling the gel. A gray precipitate on the gel may be owing to insufficient washing.

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Proteolytic Activity Detection by Two-Dimensional Zymography

Jeff Wilkesman

1. Introduction

Proteases, also known as proteinases, proteolytic enzymes or peptidases, are enzymes that hydrolyze the peptide bond of proteins (generally peptide bonds); hence, they are all hydrolases. Proteases are normally generated as an inactive proenzyme (zymogene), and according to requirements, converted into the active form through limited proteolysis. Proteolytic enzymes are ubiquitous, being distributed in all biological fluids and tissues ***(1).***

In general, proteases accomplish two major functions: (a) a regulatory function, which involves activation or inactivation of specific proteins by selective proteolysis, and (b) a general proteolytic function, which is a less specific process, resulting in the bulk breakdown of cellular proteins. These degradative mechanisms remove denatured proteins as well as facilitate adaptive responses by destroying native proteins no longer needed by the cell. Both types of proteolysis are highly regulated ***(2).***

According to the enzyme nomenclature of the International Union of Biochemistry and Molecular Biology ***(3),*** hydrolases belong to class 3, and peptidases to the subclass 3.4. This subclass can be divided depending on the type of reaction catalyzed into: (a) exopeptidases or peptidases, which are proteases catalyzing the splitting of peptide bonds at either the N- or C- terminus of the substrate (EC 3.4.11-17.X); and (b) endopeptidases or proteinases, which are proteases splitting the peptide bonds within the protein substrate (EC 3.4.21-24.X). The term protease is used only when the specificity and mechanism of hydrolysis of an enzyme are unknown ***(4).*** Endopeptidases are further classified, according to their active site, into serine, cysteine, aspartic or metallo-endopeptidases.

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The analysis of proteases by two-dimensional zymography has not been studied thoroughly, still some articles had been published ***(5, 6, 7, 8, 9).*** Widespread is, however, the study of serine and metalloproteases by one-dimensional zymography (***10, 11, 12, 13, 14,*** among many others). This chapter focuses on the analysis of protease activities by applying a simple methodology consisting on an isoelectric focusing, followed by a gel electrophoresis with a substrate copolymerized, often called zymogram. The net technique is called two-dimensional zymography, giving valuable information about the enzymatic activity, as M_r and p*I*, without the need to purify the enzyme to a high degree. In order to correlate spots pattern obtained in the zymogram, a parallel run of the sample on a normal SDS-PAGE (2D-electrophoresis) is strongly recommended.

The general methodology implies first to prepare the sample appropriately (low ionic strength, no heat treatment), then the sample is submitted to isoelectric focusing using IPG (immobilized pH gradients) strips, which generally over 12 h. During this time, SDS-PAGE and zymogram gels are polymerized. After focusing, IPG-strips must be equilibrated prior submitting them to the second dimension. Once the strips are equilibrated, they are put over the gel matrix of the zymogram and run. Subsequently, the zymogram is washed to remove the excess of SDS that might interfere with enzymatic activity, and afterwards, incubated in an activation buffer for enzyme (partial) renaturalization. Finally, zymograms can be stained with Coomassie blue. Enzymatic activity is visualized as translucent spots over a deep blue background.

2. Materials

2.1. Reagents and Equipment

1. Reagents: Tris-HCl, SDS, acrylamide:bisacrylamide mix 29:1, gelatin, TEMED, $CaCl_2$, Tween-20, Coomassie brilliant blue G-250, ethanol, 2-propanol, acetic acid, glycerol, bromophenol blue (BrPhBlue), agarose, molecular weight standards (for 1 and 2-D), urea, CHAPS, DTT, Bio-Lytes™ (Ampholytes), IPG-strips for IEF, iodoacetamide (IAA).
2. Equipment: Electrophoresis gel apparatus, power supply, incubator, shaker, isoelectric focusing apparatus.

2.2. Sample Preparation

1. Buffer A: 8 M urea, 1% CHAPS, 10 mM DTT, 0.25% Bio-Lytes™ (Ampholytes) 3–10, final volume including sample: 250 µL (*see* **Note 1**). Solution must be fresh prepared.

2.3. SDS-PAGE Preparation

1. **Table 1** shows the components needed for gel preparation for normal (substrate-free) SDS-PAGE and for gelatin copolymerized zymogram.

Table 1
Preparation of SDS-PAGE and Zymogram Minigels (8×6 cm²)

Stock solution component	Stock concentration	Stability of stock	Volume (mL)	
			7.5% SDS-PAGE	7.5% Zymogram
H_2O (d)	-	-	2.45	1.95
Acrylamide mix	30%	for months at 4°C	1.25	1.25
Tris-HCl pH 8.8	1.5 M	for months at 4°C	1.25	1.25
APS	10%	15–30 days at 4°C	0.05	0.05
TEMED	100%	for months at 4°C	0.004	0.004
Gelatin*	1%	one month at 4°C, one year at −20°C	-	0.5
		Final volume	5 mL	5 mL

see* **Note 2

Table 2
Preparation of Equilibration Buffers

Buffer component	Stock concentration	Final concentration	Buffer	
			B	C
Urea	-	6 M	1.8 g	1.8 g
Tris-HCl pH 8.8	1.5 M	0.375 M	1.25 mL	1.25 mL
SDS	10%	2%	1 mL	1 mL
Glycerol	100%	20%	1 mL	1 mL
DTT	-	2%	0.1 g	-
IAA	-	2.5%	-	0.125 g
BrPhBlue	1%	BrPhBlue	-	25 µL
		Final volume	5 mL	5 mL

2.4. Strips Equilibration for SDS-PAGE

1. **Table 2** shows the components needed for the elaboration of buffer B and C for strips equilibration. Buffers must be prepared immediately before use.
2. Running buffer: 196 m*M* glycine, 0.1% (w/v) SDS, 50 m*M* Tris pH 8.3.

2.5. Zymogram Treatment and Staining

1. Washing solution: 2.5 % (v/v) Triton X-100 (stable for several months at 4°C).
2. Activation buffer, Buffer D: 0.1 *M* Tris-HCl pH 8.0, 0.01 *M* $CaCl_2$ (stable for several months at 4°C).
3. Staining solution: 0.5 % Coomassie Brilliant Blue G-250, 40% ethanol, 10% acetic acid (stable for several months at room temperature. Can be repeatedly reused).

4. Destaining solution: 40% ethanol, 10% acetic acid (stable for several months at room temperature. Can be recovered and reused if active charcoal is added after use. A clear solution is obtained after 2–3 weeks).

3. Methods

3.1. Sample Preparation

1. Adjust the conditions of your sample according to purity and ionic strength (*see* **Note 3**).
2. Select an amount of sample to apply (active rehydration conditions are recommended). Prepare 5–100 µg protein dissolved in buffer A (*see* **Note 4**).
3. Centrifuge sample briefly.
4. Rinse IEF-focusing tray with water, 2-propanol, and water again, and then dry carefully.
5. Pipette a determined volume of sample into the line of the tray (*see* **Note 5**).
6. Select a strip with the appropriated characteristics for your sample. For easier manipulation following characteristics (for the first try) are recommended: length 7 cm, thickness: 0.5 mm (hydrated), pH range: 3–10. Remove the IPG-strip cover sheet carefully.
7. Place the IPG-strip with the gel side down in the tray (*see* **Note 6**).
8. Overlay with mineral oil (500 µL). Clean the walls of the tray with a soft paper napkin or cotton hyssops.
9. Rehydrate the strip under active conditions: 50 V, 20°C, for 10–12 h, with maximal amperage of 50 µA per strip.

3.2. Focusing

1. Saturate 2 wicks per strip with water and blot the excess of water.
2. Place wicks over electrodes.
3. Design the running program. For a 7 cm strip, theoretically the recommended program is showed in **Table 3.** A final [V•h] range between 8,000 and 35,000 should be achieved (*see* **Note 7**).

3.3. PAGE preparation

1. Prepare gels (1 mm thickness) according to **Table 1.** Pour gel solution into the glasses. No comb is needed (*see* **Note 8**). Leave a ~1 mL space for placing the strip. Cover top of gel with water. Polymerization occurs in ~30 min.

Table 3
Running Program for Focusing

Step	Voltage (V)	Time	T (°C)
1. Conditioning	250	15 min	20
2. Ramping	250 → 4000	2 h	20
3. Final focusing	4000	5 h	20

3.4. IPG-Strips Equilibration for SDS-PAGE

1. Prepare equilibration buffer B and C in 6-mL tubes according to **Table 2** (*see* **Note 9**).
2. With the forceps, carefully take strips away from the focusing tray and eliminate the excess of mineral oil by passing the strip cautiously over a napkin. Wash the strip briefly with water (d).
3. Incubate strip in buffer B for 10–15 min with constant orbital shaking. For this purpose employ 10- or 15-mL test tubes.
4. Remove strip from buffer B and incubate it now in buffer C for the same time, with gently shaking at RT.
5. While incubation is being held, heat a 1% agarose solution (*see* **Note 10**) up to 80°C.
6. While incubation, begin to prepare the gels for strip application. Wash the gels overlay with water and carefully dry the space in between the glasses with filter paper.
7. Once the surface is dried, remove the strip from buffer C and wipe the excess of liquid with a napkin. Now insert the strip between the glasses, taking special care not to touch the surface of the acrylamide gel (*see* **Note 11**).
8. Pour now carefully the agarose solution over the strip (*see* **Note 12**). Avoid any kind of bubble formation. Write down the position of the strip (*see* **Note 13**). Polymerization should occur within 15 min.
9. If wished, and if a well is available, apply molecular weight standards (*see* **Note 14**).
10. Ensemble the electrophoretic unit. Add the running buffer and run gel at constant 70 V, 4°C with maximal amperage of 40 mA for both gels, until the front dye reaches the bottom of the gel. Do not let the front leave the gel.

3.5. Zymogram Treatment and Staining

1. After run, incubate the zymogram 2 times for 30 min each with 2.5% (v/v) Triton X-100 solution, and then 3 times for 5 min with water (d) (*see* **Note 15**). The substrate-free gel run can be directly stained (by Coomassie or silver stain, according to the amount of sample added at the beginning).
2. Leave the zymogram overnight in buffer D (*see* **Note 16**).
3. Wash zymogram gels 3 times for 5 min with water.
4. Stain zymogram with a 0.5% Coomassie solution. Staining time is about 1 h. Afterwards, add destaining solution. Destain until white spots, corresponding to enzymatic activity, appear over a deep blue background. Fig. 1 shows an example of a proteolytic activity after 2-D zymography. Determination of p*I* and M_r can be now performed with specialized software available from several manufacturers. Comparisons can be hold with parallel runs done with molecular weight standards for 1-D and 2-D electrophoresis.

4. Notes

1. Concentrations of buffer components may vary, according to sample characteristics. Urea solubilizes proteins but also denatures it. Up to 9.8 M urea may be used. The use of thiourea instead of urea has also been proposed (15). Non-ionic or zwitterionic detergents are recommended in concentrations range from 0.5 until 4%. Concentration of carrier ampholytes can also be

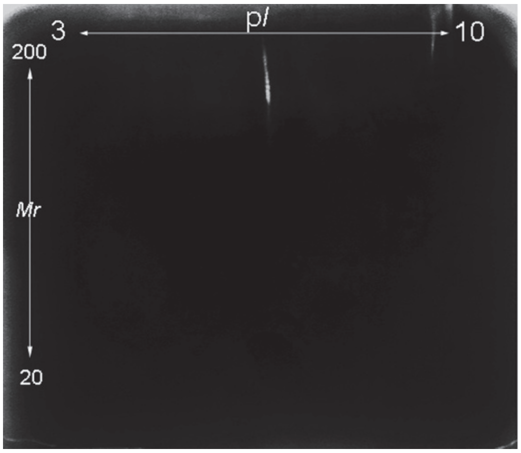

Fig. 1. 2-D zymogram of an unknown sample with proteolytic activity. About 25 µg of (total) protein was added. A p*I* ~7 and 145 kDa were determined using molecular weight standards run on a separate gel. A 10% SDS-PAGE was used copolymerized with 1% gelatin.

varied in the range 0.1 – 0.25%. Up to 100 mM DTT can also be used. Consult the instruction manual of the manufacturer of the IPG-strips in order to review the best conditions for the sample to be analyzed. It is strongly recommended to test each buffer component over the enzyme activity, as proposed by Wilkesman et al. ***(6)***.

2. Other suitable substrates besides gelatin are casein or hemoglobin. However, gelatin is highly recommended for giving very good and reproducible results. Substrate must be selected according to the type of protease to be analyzed.
3. The sample does not have to be previously purified. Experiments carried with unpurified sample should be done in order to avoid extra purification work. However, depending on the sample, some minor purification process should be done to avoid loss of biological activity, due to the presence of natural inhibitors or concomitant proteases in the biological matrix. To guarantee a good run, the protein sample must be basically salt free, with very low buffer concentration (~50 mM Tris-HCl or less)], otherwise, the strip might burn during IEF. Active rehydration is preferred as high molecular weight proteases are improved to enter into the IPG-strip ***(16)***.
4. Weigh all solids in 1.5 mL caps. Dissolve solids first in water and then add sample. Do NOT vortex the sample. Avoid bubble formation. Dissolve solids by warming the tube with the hand and with gentle up and down movements. Do NOT heat up the sample. When dissolved, let stay over ice.
5. According to strip size and tray size, volumes vary (~130 µL for 7 cm strip). Consult the instruction manual of your equipment. The mini-tray e.g., has a

minimal volume of 125 μL and a maximal of 250 μL). Use gloves for all of the following steps.

6. Avoid bubbles at this stage as best as possible. Remember (+) side over anode (red/+). Be careful with strip identification.
7. Variations are possible. For example, **Table 4** includes the rehydration step and a second conditioning step.
8. Some companies offer special combs for 2-D electrophoresis. These are useful, but not mandatory. Ready-to-use gels for 2-D purposes are also purchasable.
9. Buffers must be freshly prepared.
10. Prepare the 1% agarose solution in water or running buffer.
11. Optional: if you have not used a special comb, you may insert at this point a "tooth" of a comb to run MW markers in parallel. For easier manipulation and later interpretation, it is recommended to insert acidic (+) extreme of the strip on the left side of the gel. Remember at this step to identify which glasses contain the substrate-free gel, and which the zymogram (!).
12. Agarose temperature should be close to 80°C. A lower temperature will cause agarose to polymerize, a higher temperature could melt the polyacrylamide matrix of the gel.
13. It is recommend to put the acidic (+) extreme of the strip beside the molecular weight markers, for later identification.
14. Some companies offer special protease-molecular-weight-standards. The presence of standards in the gel is not mandatory, especially if a parallel 2-D electrophoresis is being done. Remember that normal MW standards are useless, as the whole gel will stain blue, for gelatin was copolymerized.
15. The objective to incubate with Triton is to eliminate by displacement the SDS present in the gel. SDS may inhibit protease activity. However, some proteases are detergent-resistant. If this is the case, you may jump this step.
16. This is an activation buffer. Components of the buffer, as well as incubation time, may vary according to particularities of the enzyme. These factors must be determined according to enzymatic characteristics.

Table 4
Alternative running program for focusing

Step	Voltage (V)	Time (h)	T (°C)
Active rehydration	50	12.5	20
1st Conditioning	200	2	20
2nd Conditioning	500	2	20
Ramping	500→4000	1	20
Final focusing	4000	3	20

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PART III

BLOTTING AND DETECTION METHODS

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Protein Blotting by Electroblotting

Mark Page and Robin Thorpe

1. Introduction

Identification of proteins separated by gel electrophoresis or isoelectric-focusing is often compounded by the small pore size of the gel, which limits penetration by macromolecular probes. Overcoming this problem can be achieved by blotting the proteins onto an adsorbent porous membrane (usually nitrocellulose or diazotized paper), which gives a mirror image of the gel ***(1)***. A variety of reagents can be incubated with the membrane specifically to detect and analyze the protein of interest. Antibodies are widely used as detecting reagents, and the procedure is sometimes called Western blotting. However, protein blotting or immunoblotting is the most descriptive.

Electroblotting is usually preferred for immunoblotting in which proteins are transferred to the membrane support by electrophoresis ***(2)***. A possible exception to this is in transfer from isoelectric focusing gels, where the proteins are at their isoelectric points and uncharged. Therefore, there is considerable delay before the proteins start to migrate in an electric field; also they can migrate in different directions and their rate of transfer can vary. For these reasons, transfer from isoelectric focusing gels by capillary blotting (*see* Chapter 60) may be preferable, particularly if very thin gels are used.

Electroblotting has the advantage that transfer takes only 1–4 h, and lateral diffusion of proteins (which causes diffuse bands) is reduced. Overall, nitrocellulose membranes are recommended. These are efficient protein binders and do not require activation, but are fragile and need careful handling. Nitrocellulose membranes, such as Hybond-C extra, are more robust. If the antigens of interest do not bind efficiently or if the blot is to be reused, then diazotized paper, or possibly nylon membranes, can be used. A suitable electrophoretic transfer chamber and power pack are required; these are available commercially or can be made in a laboratory work shop. The apparatus consists of a tank containing buffer, in which is located a cassette, clamping the gel and membrane tightly together.

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A current is applied from electrodes situated at either side of the cassette. The buffer is often cooled during transfer to avoid heating effects.

2. Materials

1. Transfer apparatus.
2. Orbital shaker.
3. Nitrocellulose sheet, 0.45-μm pore size, e.g., Hybond-C extra.
4. Filter paper, Whatman 3 MM (Maidstone, UK).
5. Plastic box large enough to hold the blot and allow movement on agitation.
6. Transfer buffer: 0.025 *M* Tris, 0.052 *M* glycine, 20% methanol.
7. Blocking buffer: PBS (0.14 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4) containing 5% dried milk powder.

3. Method

1. Assemble transfer apparatus and fill the tank with transfer buffer (*see* **Note 1**). If a cooling device is fitted to the apparatus, switch on before it is required to allow the buffer to cool down sufficiently (to ~8–15°C).
2. Cut two pieces of filter paper to the size of the cassette clamp, soak in transfer buffer, and place one on the cathodal side of the cassette on top of a wetted sponge pad (*see* **Note 2**).
3. Place the gel on the filter paper covering the cathodal side of the cassette (*see* **Note 3**). Keep the gel wet at all times with transfer buffer. Soak the nitrocellulose sheet (cut to the same size as the gel) in transfer buffer, and place it on the gel, i.e., on the anodal side (*see* **Note 4**). Avoid trapping air bubbles throughout the process.
4. Place the remaining filter paper over the nitrocellulose, and expel all air bubbles between the nitrocellulose and gel. This is achieved by soaking the gel/nitrocellulose/filter paper assembly liberally with transfer buffer and then pressing with a small hand roller.
5. Finally, place a wetted sponge pad on top of the filter paper, and clamp securely in the cassette. This should be a tight fit (*see* **Note 5**).
6. Place the cassette in the tank and fit the lid. Recirculate the transfer buffer either by a recirculating pump or a magnetic stirrer.
7. Electrophorese for 1–4 h at 0.5 A (*see* **Note 6**).
8. Turn off power, remove nitrocellulose sheet, and agitate in 50–200 mL of PBS containing 5% dried milk powder (*see* **Note 7**).
9. Process nitrocellulose sheet as required (*see* Chapters 71–80).

4. Notes

1. Methanol prevents polyacrylamide gels, removes SDS from polypeptides, and enhances the binding of proteins to the membrane, but it reduces the efficacy of transfer of larger proteins. It can be omitted from the transfer buffer with no adverse effects, but this has to be established empirically. Methanol is not necessary for non-SDS gels or isoelectric focusing gels.

2. Cassette clamps are normally provided with two sponge pads that fit either side of the gel/membrane/filter paper assembly to fill any dead space to squeeze the gel and membrane tightly together during electrophoresis.
3. Handle the gel and nitrocellulose sheet with gloved hands to prevent contamination with finger-derived proteins.
4. Cut a small piece of the bottom corner of the gel and nitrocellulose to orient the assembly.
5. If the gel and nitrocellulose are not clamped tightly together, then the proteins migrating from the gel can move radially and give a smeared blot.
6. Higher current can be used, but may result in uneven heating effects and blurred or distorted blots. The use of lower currents is not recommended, since transfer efficiency is reduced and poor quality blots are obtained. Overnight transfer can be used, but is not generally recommended. The time required for efficient transfer depends on the acrylamide concentration, gel thickness, gel buffer system, and the molecular size and shape of the proteins. Most proteins will pass through the nitrocellulose sheet if transfer is continued for too long. Proteins migrate faster from SDS gels (they are coated with SDS and highly charged), and transfer from non-SDS gels takes longer (around 4–5 h).
7. Other proteins can be used for blocking (e.g., albumin, hemoglobin), but dried milk powder is usually the best option. Thirty-minute incubation with blocking protein is sufficient to saturate all the protein binding sites on the blot. Longer times and overnight incubation can be used if this is more convenient. Protein blocked sheets can be stored frozen for long periods. For this, drain excess blocking solution and place in a plastic bag when required, wash with blocking buffer for 10 min, then continue with the next processing steps.

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Protein Blotting by the Semidry Method

Patricia Gravel

1. Introduction

Protein blotting, also known as Western blotting, refers to the transfer of proteins to an immobilizing membrane. The most widely used blotting method is the electrophoretic transfer of resolved proteins from a polyacrylamide gel to a nitrocellulose or polyvinylidene difluoride (PVDF) sheet and is often referred to as "Western blotting." Electrophoretic transfer uses the driving force of an electric field to elute proteins from gels and to immobilize them on a matrix. This method is fast, efficient and maintains the high resolution of the protein pattern ***(1).*** There are currently two main configurations of electroblotting apparatus: (1) tanks of buffer with vertically placed wire (*see* Chapter 58) or plate electrodes and (2) semidry transfer with flat-plate electrodes.

For semidry blotting, the gel and membrane are sandwiched horizontally between two stacks of buffer-wetted filter papers which are in direct contact with two closely spaced solid-plate electrodes. The name semidry refers to the limited amount of buffer which is confined to the stacks of filter paper. Semidry blotting requires considerably less buffer than the tank method, the transfer from single gels is simpler to set up, it allows the use of multiple transfer buffers (i.e different buffers in the cathode and anode electrolyte stacks) and it is reserved for rapid transfers because the amount of buffer is limited and the use of external cooling is not possible. Nevertheless, both techniques have a high efficacy and the choice between the two types of transfer is a matter of preference.

Once transferred to a membrane, proteins are more readily and equally accessible to various ligands than they were in the gel. The blot (i.e., the immobilizing matrix containing the transferred proteins) is therefore reacted with different probes, such as antibody for the identification of the corresponding antigen, lectin for the detection of glycoproteins or ligand for the detection of blotted

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receptor components, as well as for studies of protein-ligand associations (*see* **ref 2**). The blot is also widely used with various techniques of protein identification, from which the measurement of protein mass (by mass spectrometry using nitrocellulose or PVDF membrane), or the determination of the protein sequence (N-terminal Edman degradation, C-terminal sequence or amino acid analysis). Recently, a novel approach has been described for the sensitive analysis and identification of proteins, separated by 2-D PAGE, transferred onto a PVDF membrane, incubated with succinic acid and then scanned with IR-MALDI-MS (infrared matrix-assisted laser desorption/ ionisation - mass spectrometer). They demonstrated that the sensitivity for protein detection was comparable if not better than that of sensitive silver stained gels ***(3).***

The blot analysis generally requires small amounts of reagents, the transferred proteins on membrane can be stored for many weeks prior to their use and the same blot can be used for multiple successive analyses. For reviews on the basic principles involved in performing protein blotting and for an overview of some possible applications, see articles by Garfin et al. ***(1),*** Beisiegel ***(4),*** Gershoni et al. ***(5),*** Gravel ***(6),*** and Wilkins et al. ***(7).***

In the following sections, we describe a protocol for semidry blotting which uses a simple buffer system ***(8).*** The efficacy of this method is illustrated in **Fig. 1** with human plasma proteins separated by two-dimensional polyacrylamide gel

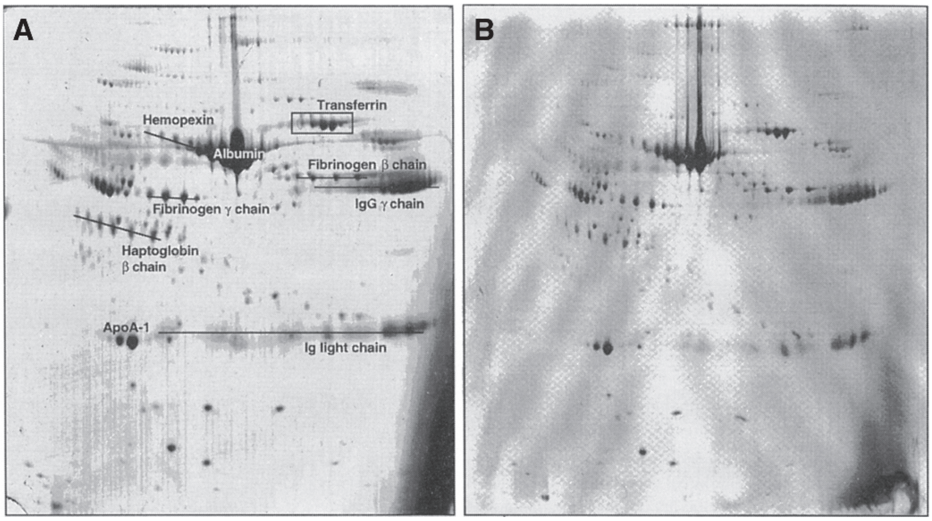

Fig. 1. Plasma proteins separated by two-dimensional polyacrylamide gel electrophoresis and (A) stained with Coomasie Brilliant Blue R250 or **(B)** transferred to PVDF membrane using the semidry system (2h, 15V) with Towbin buffer diluted 1:2 in water and stained with Coomasie Blue.

electrophoresis (2-D PAGE), transferred on PVDF membrane and stained with coomasie blue. The blot pattern is compared to the coomasie blue staining of the same protein sample before transfer from 2-D PAGE. The resolution, shape and abundance of protein spots on membrane are comparable to the 2-D polyacrylamide gel pattern. This blotting procedure allows a good and almost complete elution of proteins from the gel and their immobilization on the membrane. We will also review in **Subheading 4** the principal types of transfer matrix and the different discontinuous or continuous buffers that can be used with this technique.

2. Materials

1. Buffer: The transfer and equilibration buffer is the Towbin buffer diluted 1:2 with distilled water: 12.5 m*M* Tris, 96 mM glycine and 10% (v/v) methanol (9) (*see* **Note 1** and **Table 1**).
2. Membranes and filter papers: PVDF (0.2 µm, 200 × 200 mm, Bio-Rad) or nitrocellulose (0.45 µm, Schleicher & Shuell). Filter papers are chromatography papers (grade 3 MM CHr, Whatman). Thicker blotting papers can also be used (Pharmacia-LKB, filter paper for blotting, 200 × 250 mm).
3. Electroblotting apparatus: Proteins are transferred with a Trans-Blot SD semidry cell (Bio-Rad). The anode of the apparatus is made of platinum-coated titanium and the cathode is made of stainless steel. The maximum gel size that can be used with this apparatus is 25 cm × 18.5 cm.
4. Coomasie staining: Proteins in the 2-D gel and on the blot **(Fig. 1)** are stained with a 0.025% (w/v) solution of coomasie brilliant blue R250 solubilized in 43% (v/v) methanol and 7% (v/v) acetic acid. The destaining solution contains 30% (v/v) methanol and 7% (v/v) acetic acid.

All chemicals and methanol are of analytical reagent grade. Metallic contaminants in low grade methanol normally deposit on the electrodes.

3. Method

Note: *To avoid membrane contamination, always use forceps or wear gloves when handling membranes.*

3.1. Preparation for Semidry Blotting

1. Prepare the transfer buffer the day preceding the blotting experiments and store it at 4°C.
2. After the separation of proteins by SDS-PAGE or 2-D PAGE, the gel is briefly rinsed in distilled water for a few seconds and then equilibrated in transfer buffer for 10 minutes under gentle agitation (*see* **Note 2**).
3. During the equilibration, the filter paper and the transfer membrane are cut to the dimensions of the gel. Six pieces of filter paper per gel are needed for each gel/membrane sandwich (or two pieces of thick filter papers).

4. If the hydrophobic PVDF membrane is used, it should be prewetted prior to equilibration in transfer buffer. Immerse the membrane in a small volume of 100% methanol for a few seconds, until the entire membrane is translucent, rinse it in deionized water and then equilibrate it in transfer buffer for 3–5 minutes. It is important to keep the membrane wet at all times since proteins will not bind to the dried PVDF membrane (*see* **Note 3**).

   For hydrophilic nitrocellulose membrane, wet it in transfer buffer directly and allow it to soak for approximately 5 minutes (*see* **Notes 4** and **5** and **Table 2** for the description of the different transfer membranes).
5. If multiple full-size gels are to be transferred at one time, there is a necessity to interleave a dialysis membrane between the gel-membrane pairs to prevent proteins being driven through membranes into subsequent stacks.

   Cut a piece of dialysis membrane with the appropriate molecular weight cutoff to the dimensions of the gel and soak it in the transfer buffer (*see* **Note 6**).

3.2. Assembly of the Semidry Unit

The assembly of a semidry electroblotting apparatus is represented in **Fig. 2**. Four minigels can also be transferred at the same time by placing them side-by-side on the anode platform.

1. The filter papers are briefly soaked in transfer buffer for a few seconds.
   Place a prewetted filter paper onto the anode. Use a pipette or a painter roller to eliminate all air bubbles by pressing firmly all over the area of the paper (*see* **Note 7**). If a thin filter paper is used, add two more sheets and remove air bubbles between each layer.
2. Place the preequilibrated transfer membrane on top of the filter paper.
3. Place the equilibrated gel on top and on the center of the membrane.
4. Place another wetted sheet of filter paper on top of the gel. If a thin filter paper is used, add 2 additional sheets. Roll out air bubbles.
5. Place the cathode onto the stack.

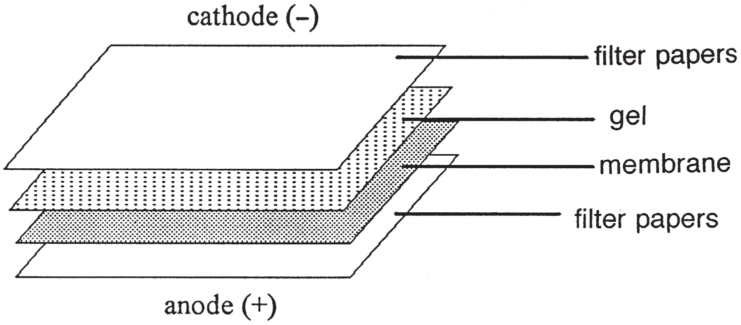

Fig. 2. Assembly of a horizontal electroblotting apparatus.

6. The blotting unit is then connected to a power supply and proteins are transferred for 2 h at a constant voltage of 15 V and at room temperature.
7. After protein blotting, the membrane is rinsed (3 × 5 min) with distilled water and is then ready for blot analysis. Following transfer, the first step for blot analysis is to block unoccupied binding sites on the membrane to prevent non-specific binding of probes, most of which are proteins (antibody, lectin). *See* **Note 8** and **Table 3** for a description of the blocking procedure.
8. The blots can be stored for many weeks prior to their use. *See* **Note 9** for storage conditions.

4. Notes

1. Since the introduction of western blotting in 1979 by Towbin et al. ***(10),*** many other buffer systems have been developed in order to improve electrophoretic transfer of proteins. The most common systems used with semidry apparatus are listed in **Table 1**.

   The two critical factors during transfer are the elution efficacy of proteins out of the gel and the binding capacity of the matrix for proteins. The elution efficacy is mainly determined by the acrylamide concentration of the gel, the ionic strength, the pH of the buffer and additional constituents of the transfer buffer such as sodium-dodecyl-sulfate (SDS) and methanol.

   The binding capacity is mainly determined by the character of the membrane but also by the transfer buffer composition ***(5).***

   Alkaline pH and SDS favour the elution of the proteins from the gel, whereas acidic pH and methanol favour their adsorption on the negatively charged membrane ***(19).*** Methanol increases the binding capacity of matrix presumably by exposing hydrophobic protein domains, so that they can interact with the matrix. Also, methanol decreases the elution efficacy by fixing the proteins in the gel and by reducing the gel pore size ***(1, 5).*** When there is SDS in transfer buffer (up to about 0.1% w/v), the proteins are negatively charged and elute efficiently from the gel.

   Semidry conditions for blotting allow the use of multiple transfer buffers (discontinuous buffer systems) to ensure a faster and better electrotransfer ***(18).*** Examples of discontinuous buffer systems are listed in **Table 1**. However, Bjerrum and Schafer-Nielsen ***(13)*** showed that there is no advantage in using different buffers in the cathode and anode electrolyte stacks. They found comparable transfer efficiencies for semidry blots performed in continuous and discontinuous buffers. We tested different buffer systems (Towbin buffer; Towbin buffer diluted 1:2 with water and Laurière buffer) for transferring plasma proteins from 2-D PAGE by semidry method and we similarly found no advantage in using discontinuous buffer systems (unpublished results).
2. Rinsing and equilibrating the gel facilitate the removal of electrophoresis buffer salt and excess of detergent. If salts are still present, the conductivity of the transfer buffer increases and excessive heat is generated during the transfer. Also, an equilibration period allows the gel to adjust to its final size prior to electroblotting because the gel shrinks in methanol-containing transfer buffer.

The duration of equilibration depends on the gel thickness. For a 1.5 mm thick gel, 10 minutes of equilibration are enough.

Recently, poly(ethylene glycol) polymers (PEG 1000–2000) have been used to complete electroblotting, in order to obtain better resolution and enhancement of sensitivity of proteins on membrane. PEG reduces background, raises signal-to-noise ratio and sharpens protein band ***(20).*** After polyacrylamide gel electrophoresis, 30 % (w/v) PEG (solubilized in transfer buffer: 12.5 mM Tris, 96 mM glycine and 15% (v/v) methanol) is applied to reversibly fix proteins within the gel. The intragel proteins can then be electroblotted directly onto membranes using the same transfer buffer containing PEG.

3. If the PVDF membrane does dry out during use it can be rewet in methanol. Membranes that contain adsorbed proteins and that have been allowed to dry can also be wet again in methanol. In our experience, we have not seen any difficulty in protein staining, glycoprotein detection and immunostaining after this re-wetting procedure in methanol.
4. Nitrocellulose membrane was the first matrix used in electroblotting ***(10)*** and is still the support used for most protein blotting experiments. It has a high binding capacity, the non-specific protein binding sites are easily and rapidly blocked, allowing low background staining and it is not expensive. For blotting with mixtures of proteins, standard nitrocellulose with a pore size of 0.45 µm should be used first. However, membranes having 0.1 µm and 0.2 µm pore sizes should be tried if some low-molecular weight proteins do not bind efficiently to the standard membrane ***(1).*** After staining, the nitrocellulose membranes become transparent simply by impregnating the membrane with concentrated Triton X-114 ***(21).*** The blot can thus be photographed by transillumination or scanned with a densitometer for quantitative analysis. Long term stability (several months) of transparent stained blots is possible if they are stored at −20°C.

PVDF membranes are more expensive but have high mechanical strength, high protein binding capacity and are compatible with most commonly used protein stains and immunochemical detection systems. The chemical structure of the membrane offers excellent resistance to acidic and organic solvents. This makes PVDF membrane an appropriate support for N-terminal protein sequencing and amino acid composition analysis. Another interesting advantage of PVDF matrix over the nitrocellulose is the possibility to visualize the protein pattern on the blot without staining. After blotting, the PVDF membrane should be placed on top of a vessel containing distilled water. The immersion of the membrane in water should be avoided. It should be laid down at the surface. The protein spots (or bands) contrast with the remainder of the membrane and can be visualized. To obtain a clear image of the protein pattern, the surface of the membrane should be observed from different angles and under appropriate lighting. This procedure, which was found unintentionally, is easy to perform and allows rapid and good evaluation of the transfer quality.

Table 2 summarizes the most common matrices that can be used for transferring proteins from polyacrylamide gels.

5. Whatever the membrane used, exceeding its binding capacity tends to reduce the signal eventually obtained on blots. It can be assumed that excess protein, weakly associated with the membrane, may be readily accessible to react with the probe in solution, but the probe-protein complexes formed may then be easily washed off during the further processing of the membrane ***(5).*** This situation does not occur if the proteins are initially in good contact with the membrane.

   For 2-D PAGE, the best recovery and resolution of proteins are obtained when loading 120 µg of human plasma or platelet proteins. When 400 µg of protein are separated by 2-D PAGE and transferred on membrane, the spots are diffused and the basic proteins poorly transferred (not shown). This could be attributed to overloading which prevents a good separation of proteins and an adequate binding to transfer matrix.
6. It is very difficult to form large stacks of gel-membrane pairs. Even 2 pairs are often associated with the introduction of air bubbles. We prefer to use a semidry unit to transfer proteins from a single gel only.
7. Air bubbles create points of high resistance and this results in spot (or band) areas of low efficacy transfer and spot (or band) distortion.
8. The quality or extent of the blocking step determines the level of background interference. It has been recognized that the blocking step may also promote renaturation of epitopes ***(23).*** This latter aspect is particularly important when working with monoclonal antibodies (which often fail to recognize the corresponding antigenic site after electroblotting). Hauri and Bucher ***(23)*** suggest that monoclonal antibodies may have individual blocking requirements, probably due to different degrees of epitope renaturation and/or accessibility of antibody under the various blocking conditions.

   Some common blocking agents are listed in **Table 3**.

   For immunoblotting on nitrocellulose membrane we obtain good results by using a blocking solution made of 0.5% (w/v) BSA, 0.2% (w/v) Tween-20 and 5% (w/v) non fat dried milk in PBS buffer (137 m*M* NaCl, 27 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4) ***(29).*** For lectin blotting on PVDF membrane, we use 0.5% (w/v) Tween-20 in PBS buffer ***(8).***
9. We store the blots at 4°C in PBS containing 0.005% (w/v) sodium azide. To evaluate the effect of storage on blotted proteins, we stained with colloidal gold a nitrocellulose blot of platelet proteins stored for a period of 4 months in PBS/azide at 4°C. We observed a protein pattern identical to the same blot stained immediately after western blotting (not shown).

   On the other hand, when we dried a nitrocellulose blot at room temperature and stained 24 hours later the proteins with colloidal gold, we observed important contaminating spots on the blots.

   Only one point should be kept in mind when storing blots in PBS/azide. Sodium azide inhibits peroxidase activity and therefore good washing of the membrane with PBS is necessary before blot analysis using probe labeled with peroxidase.

   As an alternative to blot storage, the gel can be frozen at −80°C. Immediately after electrophoresis, the gel should be rinsed in distilled water for a few seconds and placed in a plastic bag between two precooled glass plates. It is important to precool glass plates at −80°C, otherwise the gel will crack when thawed. These frozen gels can be stored at −80°C for at least 3 months ***(19)***. Before the transfer procedure, the frozen gels are thawed in their plastic bags and then equilibrated as described in Subheading 3.

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Protein Blotting by the Capillary Method

John M. Walker

1. Introduction

The ability to transfer (blot) separated proteins from a polyacrylamide gel matrix onto a sheet of nitrocellullose paper (where the proteins bind to the surface of the paper) has provided a powerful tool for protein analysis. Once immobilized on the surface of the nitrocellulose sheet, a variety of analytical procedures may be carried out on the proteins that otherwise would have proven difficult or impossible in the gel. Such procedures may include hybridization with labeled DNA or RNA probes, detection with antibodies (probably the most commonly used procedure), detection by specific staining procedures, autoradiographic assay, and so forth. The most commonly used, and indeed most efficient, methods for transferring proteins from gels to nitrocellulose (blotting) are by electrophoresis, and these methods are described in Chapters 58 and 59. An alternative method, capillary blotting, is described here. Although this method takes longer than electroblotting methods (it takes overnight) and transfer of proteins from the gel is not as complete as it is for electroblotting (although sufficient protein is transferred for most purposes) the method does have its uses. It is of course ideal for those who only wish to carry out occasional blotting experiments and therefore do not wish to commit themselves to the purchase or the purpose-built apparatus (plus power pack) needed for electroblotting. Second, this method is particularly useful for blotting isoelectric focusing gels where the proteins are at their isoelectric point (i.e., they have zero overall change) and are not easy therefore to transfer by electrophoresis. The method simply involves placing the gel on filter paper soaked in buffer. Buffer is drawn through the gel by capillary action by placing a pad of **dry** absorbent material on top of the gel. As the buffer passes through the gel, it carries the protein bands

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with it, and these bind to the nitrocellulose sheet that is placed between the top of the gel and the dry absorbent material.

2. Materials

1. Blotting buffer: 20 m*M* Tris, 150 m*M* glycine, 20% methanol, pH 8.3. Dissolve 4.83 g of Tris base, 20.5 g of glycine, and 400 mL of methanol in 2 L of distilled water. The solution is stable for weeks at 4°C.
2. Nitrocellulose paper.
3. Whatman 3 MM filter paper.
4. Absorbent material: e.g., a wad of filter paper or paper towels; this author uses baby diapers.

3. Method

1. Place two sheets of 3 MM filter paper on a glass plate, and thoroughly soak them in blotting buffer. The ends should be dipped in reservoirs containing blotting buffer to ensure this filter paper pad remains wet overnight (*see* **Fig. 1**).
2. Place the gel to be blotted on top of this filter paper bed. Make sure the bed is fully wetted, and that no bubbles are trapped between the gel and filter paper. Thoroughly wet the top of the gel with blotting buffer.
3. Cut a piece of nitrocellulose paper to the size of the gel to be blotted. Wet the paper by dipping it in blotting buffer. **(Care: Use gloves; there are more proteins on your fingers than you are blotting from the gel!)** Then lay the nitrocellulose sheet on the gel surface. Take great care to ensure no air bubbles are trapped (buffer cannot pass through an air bubble).
4. Now dry material must be placed on top of the nitrocellulose. Start with three sheets of 3 MM filter paper, cut to the same size as the gel, and then place on top of this a pad of your absorbent material (*see* **item 4, Subheading 2.**).
5. Finally, place a heavy weight on top of the absorbent rnaterial, e.g., a sheet of thick glass that supports a 2–3 L flask filled with water (*see* **Note 1**).
6. Allow blotting to take place overnight (preferably for 24 h). The setup may then be dismantled. the nitrocellulose sheet stained for protein to confirm that transfer has been achieved (e.g., with 0.2% Ponceau S in 10% acetic acid), and then the

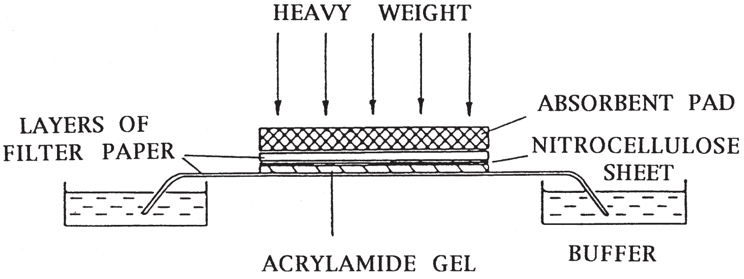

Fig. 1. A typical arrangement for capillary blotting.

nitrocellulose sheet blocked and probed using any of the methods described in Chapters 74–80.

4. Note

1. The only error you can make with this method is to have any of your dry material (e.g., filter paper or absorbance pad) overhanging the gel and making contact with the wet base of filter paper. In this case, buffer preferentially travels around the gel into the absorbant pad, rather than through the gel. When the heavy weight is applied to the setup check that this is not happening (the absorbent pad often "sags" quite easily). If there is overlap, simply trim with scissors. Do not expect the absorbent pad to be particularly wet after an overnight blot. It should be barely damp after an overnight run. If it is soaking wet, then this indicates that buffer has traveled around the gel. However, even if this is the case, it is probably worth proceeding since some protein will have transferred nevertheless and this can probably still be detected with your probe.

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Western Blotting of Basic Proteins Electrophoretically Resolved on Acid-Urea-Triton-Polyacrylamide Gels

Geneviève P. Delcuve and James R. Davie

1. Introduction

The electrophoretic resolution of histones on acetic acid-urea-Triton (AUT) polyacrylamide gels is the method of choice to separate basic proteins such as histone variants, modified histone species, and high mobility group proteins 14 and 17 (***1-6***). Basic proteins are resolved in this system on the basis of their size, charge and hydrophobicity. In previous studies, we analyzed the abundance of ubiquitinated histones by resolving the histones on two-dimensional (AUT into SDS) polyacrylamide gels, followed by their transfer to nitrocellulose membranes, and immunochemical staining of nitrocellulose membranes with an anti-ubiquitin antibody (***7-9***). However, transfer of the basic proteins directly from the AUT polyacrylamide gel circumvents the need to run the second dimension SDS gel and accomplishes the analysis of several histone samples. We have described a method that efficiently transfers basic proteins from AUT polyacrylamide gels to nitrocellulose membranes (***10***). This method has been used in the immunochemical detection of modified histones isoforms and histone H1 subtypes (***6, 11-14***).

To achieve satisfactory transfer of basic proteins from AUT gels to nitrocellulose, polyacrylamide gels are submerged in 50 mM acetic acid, 0.5% SDS (equilibration buffer 1; 2 × 30 min) to displace the Triton X-100, followed by a 30 min incubation in a Tris-SDS buffer (equilibration buffer 2). The transfer buffer is an alkaline transfer buffer (25 mM CAPS, pH 10, 20% (v/v) methanol). Szewczyk and Kozloff (***15***) reported that alkaline transfer buffers increase the efficiency of transfer of strongly basic proteins from SDS gels to nitrocellulose

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membranes. We reasoned that this transfer buffer would improve the transfer of histones from AUT gels that had been treated with SDS.

2. Materials

Buffers are made from analytical grade reagents dissolved in double distilled water.

1. Equilibration buffer 1: 0.575 ml of glacial acetic acid (50 m*M*), 10 mL of 10% (10 g in 100 mL of water) SDS (0.5%), and water to 200 mL.
2. Equilibration buffer 2: 6.25 mL of 1 *M* Tris-HCl (62.5 mM), pH 6.8, 23 mL of 10% (w/v) SDS (2.3%), 5 mls of β-mercaptoethanol (5%), and water to 100 mL. 1 *M* Tris-HCl: 121 g of Tris base in 800 mL of water and adjusted to pH 6.8 with hydrochloric acid. The buffer is made up to 1000 mL.
3. Transfer membrane: Nitrocellulose membranes (0.45 μm pore size, Schleicher & Schuell, BA85) are used.
4. Transfer buffer: 187 mL of Caps (3-(cyclohexylamino)-1-propanesulfonic acid) stock (16X) solution (final concentration, 25 m*M*), 600 mL of methanol (20%), and water to 3 L. Caps stock solution (400 m*M*): 88.5 g of Caps dissolved in 800 mL of water and adjusted to pH 10 with 10 *M* NaOH. The buffer is made up to 1000 mL.
5. Electroblotting equipment: Proteins are transferred with the Bio-Rad Trans-Blot transfer cell containing a super cooling coil (Bio-Rad). Cooling is achieved with a Lauda circulating bath.
6. Protein stain: Proteins in the AUT or SDS polyacrylamide gel are stained with Coomassie Blue (Serva Blue G). Proteins on the membrane are stained with Indian ink (Osmiroid International Ltd.) (0.01% v/v in TBS-TW). TBS-TW: 20 m*M* Tris-HCl, pH 7.5, 500 m*M* NaCl, and 0.03% (v/v) Tween-20.

3. Method

3.1. Protein Blotting

1. Nitrocellulose membranes are cut to size of gel at least a day before transfer and stored in water at 4°C.
2. The proteins are electrophoretically resolved on AUT- or SDS-polyacrylamide minislab gels (6 mm long, 10 mm wide, 0.8–1.0 mm thick).
3. Following electrophoresis the AUT polyacrylamide gel is gently shaken in 100 mL of equilibration buffer 1 for 30 min at room temperature. This solution is poured off, and another 100 mL of equilibration buffer 1 is added. The gel is again shaken for 30 min. The solution is discarded and replaced with equilibration buffer 2. The gel is agitated in this solution for 30 min.
4. Onto the gel holder place the porous pad that is equilibrated with transfer buffer. Three sheets of 3 MM paper soaked in transfer buffer are placed on top of the porous pad. The treated AUT or SDS slab gel is placed onto the 3 MM paper sheets. Nitrocellulose membrane is placed carefully on top of the gel, avoiding the trapping of air between the gel and nitrocellulose membrane. One sheet of 3 MM paper soaked

in transfer buffer is placed on top of the nitrocellulose membrane, followed by the placement of a porous pad which has been equilibrated with transfer buffer.

5. The gel holder is put into the transblot tank with the polyacrylamide gel facing the cathode and the nitrocellulose membrane facing the anode. The tank is filled with transfer buffer. Protein transfer is carried out at 70 V for 2 h and/or at 30 V overnight with cooling at 4°C.
6. Following transfer, the nitrocellulose membrane is placed onto a sheet of 3 MM paper and allowed to dry for 30 min at room temperature. The gel is stained with Coomassie Blue. The air-dried nitrocellulose membrane is placed between two sheets of 3 MM paper. This is wrapped with aluminum foil and baked at 65°C for 30 min. The proteins transferred onto the nitrocellulose membrane may be visualized by staining the nitrocellulose membrane with India ink (*see* **Subheading 3.2**).

3.2. Protein Staining

1. The nitrocellulose membrane is agitated in TBS-TW (0.7 ml per cm^2) for 10 min at room temperature in a sealed plastic box. The solution is discarded and fresh TBS-TW is added. These steps are repeated twice more for a total of four washes of the membrane in the TBS-TW solution.
2. The India ink stain is added to the nitrocellulose membrane (0.56 ml/cm^2) which is agitated for 30 min to 2 h at room temperature.
3. The stain is discarded and the nitrocellulose membrane is shaken in water for 5 min at room temperature.
4. The nitrocellulose membranes are dried and stored.

4. Notes

1. Nitrocellulose membranes have been used in the majority of our studies. However, these membranes are fragile and must be handled with care. An alternate membrane, which is stronger than nitrocellulose, is PVDF (Bio-Rad). The PVDF membranes are wetted with 100% methanol for 3 min and then equilibrated with transfer buffer for 3 min.
2. The efficiency of transfer of basic proteins (histones) from AUT polyacrylamide slab gels to nitrocellulose membranes is shown in **Fig. 1**. Most of the histones were efficiently transferred. The efficiency of elution was poorest for histone H1. **Figure 1C** demonstrates that the histone variants of histone H2A (H2A, H2A.Z) and of histone H3 (H3.1, H3.2, H3.3) and the modified histone species (e.g., ubiquitinated histone H2A, acetylated histone H4) were transferred. Densitometric tracings of the gel patterns before and after transfer demonstrated that greater than 90% of the histones H2A, H2B, H3, and H4 and approximately 80% of histone H1 were eluted from the AUT gel.
3. The transfer efficiency of basic proteins from AUT minislab polyacrylamide gels to nitrocellulose membranes was poor when a Tris-glycine-methanol

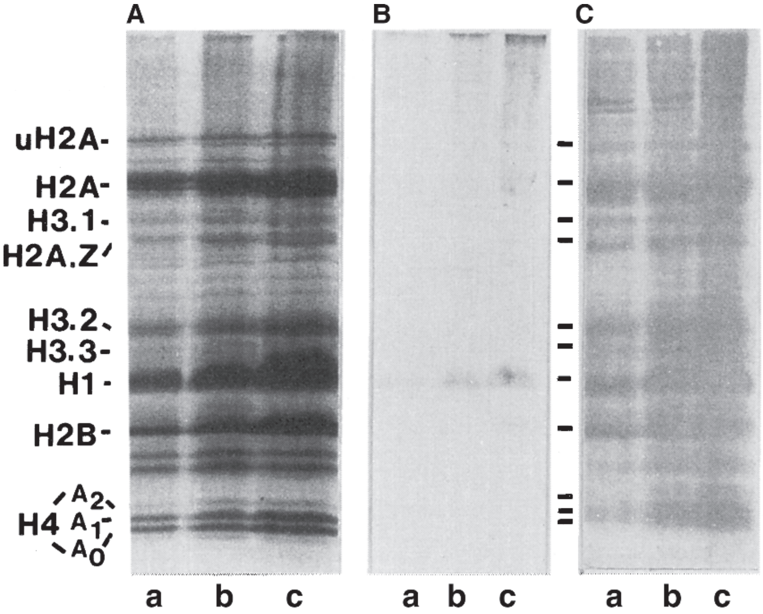

Fig. 1. Electrophoretic transfer of histones from AUT minislab gels. **(A)** Histones (9, 18 and 36 µg in lanes a, b and c, respectively) isolated from T-47D-5 human breast cancer cells were electrophoretically resolved on AUT minislab gels. The gel was stained with Coomassie Blue. **(B)** The Coomassie Blue-stained AUT minislab gel pattern of histones remaining after transfer (30 V overnight) to nitrocellulose is shown. **(C)** The India ink-stained nitrocellulose pattern of histones transferred from the AUT minislab gel in B is shown. A_0, A_1 and A_2 correspond to the un-, mono- and diacetylated species of histone H4, respectively. The ubiquitin adduct of histone H2A is denoted as uH2A. Reprinted with permission from ***ref. 10*** (copyright by the Academic Press).

(25 m*M* Tris, 192 m*M* glycine, 20% (v/v) methanol, and 0.1% SDS) transfer buffer was used.

4. With the Bio-Rad Trans-Blot cassette, four minislab gels can be easily accommodated.
5. Following transfer and baking, the nitrocellulose membrane may be stored for several weeks at room temperature before proceeding with immunochemical staining.
6. Leaving the nitrocellulose membrane in water for too long after staining with India ink will result in removal of the stain.
7. We have used this alkaline transfer buffer to transfer histones from SDS slab gels to nitrocellulose membranes. Pre-treatment of the SDS slab gel is not required. However, we have found that washing the SDS slab gel in equilibration buffer 2 for 30 min improved the efficiency of elution of the histones from the SDS gel.

Acknowledgments

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Immunoblotting of 2-DE Separated Proteins

Barbara Magi and Laura Bianchi

1. Introduction

Immunoblotting technique, also dubbed "Western blotting," exploits antibody specificity to identify target proteins among a number of unrelated protein species ***(1,2).*** Traditionally used for mapping known proteins on electrophoretically-resolved mixtures, in the proteome era immunoblotting has been combined with 2-D gel electrophoresis for rapid visualization and identification of target proteins ***(3),*** protein adducts with drugs ***(4)*** and antigenic proteins associated to pathogens, allergens as well as tumours, with patient serum used as primary antibody ***(5–9).***

In the postgenomic era, gene expression is becoming a focus of attention and it is widely accepted that one gene does not necessarily encode a single protein product, as molecular mechanisms generating different isoforms from the same gene have been described in many different organisms. Although the relation between number of genes and number of potentially encoded proteins has yet to be clarified, it is usually recognized that the number of polypeptides expressed by a genome is greater than expected on the basis of calculated gene numbers. In fact, it has been estimated that the average number of alternates spliced from the transcript of a single mammalian gene may be two to three or more ***(10)*** and that many proteins may be subject to co- and posttranslational modifications and proteolytic processing. For example, up to 20% of protein is acetylated in yeast ***(11).*** Several preliminary proteome projects have also shown that prokaryotic organisms can express more than one protein isoform from a single gene and posttranslationally modified gene products have been reported ***(12).*** Moreover, compared to completely sequenced genome of other organisms, the unexpected low number of potential protein-coding genes in the human genome makes molecular mechanisms generating different isoforms from the same gene

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particularly interesting. As shown in Scheler et al. ***(13)*** and Janke et al. ***(14),*** simultaneous 2-D gel analysis of cross-reactive protein isoforms derived from a single gene may produce very complex isoforms patterns: more than 50 and 80 2-D electrophoretic protein spots were observed using specific antibodies for HSP27 and Tau protein, respectively. 2-D immunoblotting combined with mass spectrometry-based identification methods has been widely applied to the characterization of 2-D electrophoretic cross-reactive isoforms of the same protein, e.g. due to alternative splicing, co- and/or posttranslational modifications and proteolytic cleavages ***(15-21).***

Western blotting analysis is also the method of choice for simultaneous visualization of 1-D and 2-D separated proteins sharing common epitopes related to specific post-translationally-modified aminoacids or to specific functional/structural domains, e.g. anti-phosphoresidues and anti-O-linked N-acetylglucosamine antibodies ***(22-24).*** This approach has been widely used to differentially visualize tyrosine-phosphorylation profiles in cells and tissues under different conditions ***(17,22),*** as well as for studying signal transduction pathways following stimulation ***(25).*** The dynamic and reversible nature of several known post-translational modifications makes their characterization possible only at protein level, as these features characterize mature gene products and cannot be inferred from crude genome-derived aminoacid sequence stored in sequence databases. 2-D immunoblotting is therefore a powerful method for rapid visualization of target proteins, sharing a common feature, to be identified and characterized by microchemical analysis, e.g. by mass spectrometry.

Immunoblotting techniques involve identification of protein target via antigen-antibody specific reactions. Proteins are typically separated by electrophoresis in polyacrylamide gels, then transferred (“blotted”) onto chemically resilient membranes (nitrocellulose, PVDF…) where they bind in the pattern they took in the gel. The membrane is overlaid with a primary antibody directed to the specific target, then with a secondary antibody (anti-immunoglobulin) labeled with radioisotopes, enzymes…

A semplified procedure in which protein samples are not separated electrophoretically but are spotted directly onto the membrane is called “dot blot” and is a good preliminary experiment to detect the presence of a certain antigen in a sample.

A number of related techniques for probing membranes containing transferred protein with specific ligands have been described:

- In “Far-Western blotting” the membrane is probed with another protein to detect specific protein:protein interactions ***(26-28).*** The reaction can be revealed using biotinylated or GST-tagged bait or “probe” protein followed by a streptavidin-HRP or a anti-GST-HRP chemiluminescent detection system, respectively.

- Blot overlays include the probing of membrane with various molecules to detect the presence of specific binding domains, e.g. with GTP ***(29,30)*** or proteoglycans ***(31).*** In the south- or north-western blotting the membrane is probed with DNA or RNA molecules to detect nucleic-acid binding proteins ***(32).***
- In glycoprotein detection systems the carbohydrate portions of proteins are oxidized with sodium metaperiodate to generate aldehydes that can react with hydrazides. A biotin hydrazide is used to attach biotin onto the oxidized carbohydrates and horseradish peroxidase-conjugated streptavidin is used for chemiluminescence-based detection (Glycoprotein Detection Module, Amersham Biosciences, Uppsala, Sweden).
- A recently developed technique, is high-throughput western blotting, also known as western array screening of total cell or tissue lysate. It includes polyacrylamide gel electrophoresis and electroblotting, followed by screening of the blots with hundreds of high-quality antibodies, combined in unique cocktails ***(33).***

2. Materials

2.1. Equipment

1. Blotting apparatus: transfer cell, gel holder, magnetic stirrer, refrigerated thermostatic circulator unit.
2. Power supply.
3. Rocking agitator.
4. Computing Densitometer and/or a gel and blot image acquisition system.
5. PC with a computer program for 2-D gel analysis.

2.2. Reagents

1. Distilled water.
2. Nitrocellulose membrane.
3. Transfer buffer: 25 m*M* Tris, 192 m*M* glycine, 20% (v/v) methanol. Do not adjust pH, it is about 8.3.
4. Filter paper for blotting (Whatman 17 Chr).
5. Ponceau S solution: 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid (TCA).
6. Coomassie blue solution: 0.1% Coomassie blue R-250 in 40% methanol, 1% acetic acid.
7. Destaining solution: 50% methanol.
8. Phosphate buffered Saline (PBS): 0.15 *M* NaCl, 10 m*M* NaH_2PO_4: bring to pH 7.4 with NaOH.
9. Blocking solution: 3% (w/v) nonfat dry milk in PBS, 0.1% (w/v) Triton X-100.
10. Primary antibody solution (primary antibody, appropriately diluted in blocking solution).
11. Secondary antibody solution (secondary antibody, appropriately diluted in blocking solution).
12. Washing solution: 0.5% (w/v) Triton X-100 in PBS.

13. 0.05 *M* Tris-HCl, pH 6.8.
14. Amersham Biosciences ECL (enhanced chemiluminescence) kit, cat. no. RPN 2106.
15. Saran Wrap or other cling-films.
16. X-ray films, 18 cm × 24 cm (Amersham Hyper film ECL, cat. no RPN 3103).
17. Developer and fixer for X-ray film (Developer replenisher; fixer and replenisher, 3M, catalogue numbers XAF 3 and XAD 3) (3M Italia S.p.A., Segrate, Italy).
18. Stripping buffer: 100 m*M* 2-mercaptoethanol, 2% (w/v) SDS, 62.5 m*M* Tris-HCl pH 6.8.

3. Methods

3.1. Transfer

To avoid membrane contamination wear gloves during all the steps of the experiment.

1. Prepare the transfer buffer (*see* **Notes 1-3**) and cool it to 4°C before the end of the electrophoretic run.
2. Cut to the dimension of the gel, two pieces of filter paper and one piece of nitrocellulose/gel (*see* **Note 4**).
3. Following electrophoresis (*see* **Note 5**), wash the gel in distilled water and then equilibrate it in transfer buffer. The ideal time for 1.5 mm gels is 10 to15 min (*see* **Note 6**).
4. Soak the nitrocellulose membrane for 15–20 min in transfer buffer. Also wet two “Scotch-Brite” pads/gel and filter papers in transfer buffer.
5. Assemble the “sandwich” for transfer in this order: fiber pad, filter paper, nitrocellulose, gel, filter paper, fiber pad. Remove all air bubbles between membrane and gel and between paper and gel.
6. Put the blot sandwich in the gel holder and hold it firmly, to ensure a tight contact between gel and membrane.
7. Fill the cell with transfer buffer and place a stir bar inside the transfer cell, so that the buffer is stirred during electrotransfer and temperature and conductivity are uniform during electrotransfer.
8. Place the gel holder in the transfer cell with the sandwich oriented as follows: ANODE /fiber pad, filter paper, nitrocellulose, gel, filter paper, fiber pad/ CATHODE.
9. Carry out blotting at a constant current until it reaches a total of 1.5–2.0 Å (*see* **Note 7),** refrigerating the buffer to 4°C (*see* **Note 8**) for gels of 16 × 18 cm (such as two-dimensional gels) or at constant voltage (100 V) for 1 h for minigels (*see* **Note 9**).
10. After electrotransfer, disassemble the blotting apparatus and remove the nitrocellulose membrane. To mark the orientation of the membrane, cut away the lower right corner, corresponding to low M_r, high pH.

The membrane can be processed immediately for immunoblotting or can be air-dried and stored at −20°C, within parafilm sheets for extended periods ***(34).***

Though electroblot is by far the most widely used technique, there are also alternative methods, as discussed in a recent review ***(35).***

3.2. Staining of Total Protein Pattern on Membrane

3.2.1. SDS-PAGE and IEF Gels

In the case on one-dimensional gel (IEF or SDS PAGE), staining of total protein pattern can be done on one lane of the gel in which the sample or MW standard or pI standard have been loaded. Nonreversible stains more sensitive than Ponceau Red can be used (*see* **Note 10**).

1. Cut the nitrocellulose lane to be stained and immerse it in Coomassie blue solution for 3 min.
2. Destain for 30 min destaining solution and air dry.

3.2.2. Two-dimensional gels

1. Before the immunodetection, stain the nitrocellulose membrane in 0.2% w/v Ponceau S in 3% w/v trichloroacetic acid for 3 min ***(36)*** (*see* **Note 11).**
2. Destain with several changes of distilled water to diminish background color. Because the red spots will disappear in the blocking step, save the image with a chemiluminescence image acquisition system or circle with a waterproof pen some spots before the next steps of immunostaining. Spot stained with Ponceau S will be used as landmarks to match total protein pattern on nitrocellulose against immunoreactive pattern and against silver stained polyacrylamide gel pattern (*see* **Note 11**).

3.3. Immunodetection

3.3.1. Incubation with Antibodies

All steps are carried out at room temperature and with gentle agitation on a rocking agitator.

1. Block nonspecific binding sites in the membrane with three washing steps, each 10 min in duration, in blocking solution (*see* **Notes 12, 13** and **14**).
2. Incubate overnight in the primary antibody solution at the suitable dilution (*see* **Note 15**) in blocking solution.
3. Wash 3 × 10 min in blocking solution.
4. Incubate for 2 h in the secondary antibody solution (*see* **Notes 16** and **17**).
5. Wash 3 × 10 min in blocking solution.
6. Wash 30 min in washing solution.
7. Wash 2 × 30 min in 0.05 M Tris-HCl pH 6.8.

After this step one can go forward with ECL detection (*see* **Notes 18** and **19**). Alternatively, one can choose detection with the chromogenic substrate (*see* **Note 20**).

3.3.2. Enhanced Chemiluminescent Detection

To detect the immunoreactive spot(s) with a chemiluminescent method it is necessary to wear gloves to prevent hand contact with film. We routinely use Enhanced Chemiluminescet detection (Amersham Biosciences) because it offers a bigger sensitivity and the possibility of quantification of immune reaction (*see* **Notes 18, 21,** and **22**).

Mix equal volumes of detection reagent 1 and detection reagent 2 from the Amersham ECL kit and immerse the membrane in this solution for 1 min, ensuring that all the surface of the membrane is covered with solution. The chemiluminescent signal can be acquired by a chemiluminescence image acquisition system or by the following procedure:

1. Place the membrane on a glass and cover it with a layer of Saran Wrap.
2. Cut away a corner from a piece of autoradiography film to define its orientation (*see* **Subheading 3.1, step 10**). Superimpose the autoradiography film on the nitrocellulose membrane beginning from the upper left corners. Nitrocellulose membrane and X-ray film may have different dimensions. Superimposing at the upper left corner for ECL impression will allow subsequent matching of images.
3. Expose the film for a time variable from 5 s to several minutes. It is convenient to begin with short exposure, develop the film, and then try longer exposures, if necessary.
4. Develop the film with the suitable reagents (*see* **Subheading 2.2, item 17**).

3.4. Stripping

At the end of a cycle of immunodetection, it is possible to strip the membrane with indicated solution and to carry out subsequent cycles incubating with different primary antibodies (*see* **Note 23**).

The procedure for the stripping we use is:

1. Incubate the membrane in stripping buffer at 70°C for 30 min, with occasional shaking.
2. Wash the nitrocellulose 2 × 10 min in large volumes of washing solution at room temperature.
3. Block the membrane and perform immunodetection as described in **Subheading 3.3.**

3.5. Matching

3.5.1. SDS-PAGE and IEF Gels

For SDS-PAGE gels (or IEF gels), a MW or pI standard is normally run in one lane. The following procedure is used to identify the immunoreactive band and determine its MW or pI.

1. Scan the ECL-developed film, the Coomassie-stained nitrocellulose membrane or a lane of the Coomassie-stained gel (in which standard proteins or the sample were separated) with a computing densitometer of sufficient resolution.

2. Use a graphics programme to make the images the same length (all were originally from the same gel and are therefore of the same size, but nitrocellulose shrinks when stained)
3. When the images are perfectly aligned, the immunoreactive band can be distinguished in the Coomassie stained lane and its MW or pI determined by comparison with the appropriate lane.

3.5.2. Two-Dimensional Gels

For an accurate matching process we use a computer program (*see* **Note 24**) that permits matching the digitized images, using as landmarks the spots stained with Ponceau S (*see* **Note 25**).

To perform this operation we suggest the following procedure:

1. Scan the ECL-developed film, the Ponceau S-stained nitrocellulose membrane, and the silver-stained gel of the same sample with a computing densitometer with a sufficient resolution (*see* **Notes 26** and **27**).
2. Rotate left-right the nitrocellulose membrane, with an appropriate program, in order to have the three images with the cut lower corner on the right. In fact, the nitrocellulose membrane has the spots only in one face and the scanning process generates an image with the cut lower corner placed on the left.
3. Stack together the film and nitrocellulose membrane images, aligning the upper left corners and the two corresponding borders and placing the cut lower right corner in the same orientation for both.
4. Add “manually,” with appropriate software tool, the Ponceau S spots chosen as landmarks onto the image of the ECL film.
5. Find the spots on the gel corresponding to landmarks on the film (*see* **Note 28**) and modify the size of the silver nitrate image adjusting it to the smaller one of film by the mean of adequate software. Actually the gel is larger then the film due to silver staining procedure.
6. Stack together the equalized ECL film and gel images, superimpose the landmarks carefully and run the automatic match program. This operation permits automatically highlighting the silver-stained spots paired with the immunoreactive ones present on the ECL film (*see* **Note 29**).

4. Notes

1. We perform electrotransfer from gels to nitrocellulose membrane, using a “wet” or “tank” apparatus, where the gel is submerged in a large volume of buffer during the transfer. For two dimensional gel or gel with big sizes, we use a BioRad transfer cell with 3 L of transfer buffer or an ISODALT cell (Hoefer Scientific Instruments) with 20 L of transfer buffer. ISODALT cells allows the simultaneously transfer of 5 gels. For minigels we use Mini Trans-Blot cell (7.5 × 10 cm blotting area). The transfer buffer can be used several times, if stored at 4°C.

"Semidry" electroblotters require smaller volumes of buffer, since only the membrane and filter paper have to be wet and the procedure is faster. However, the "wet" method is recommended when antigen is present in small quantities (such as low abundance spots in 2-D gels) and/or its molecular weight is high ***(37).*** It offers more options, such as temperature, time and voltage control.

2. The transfer buffer we use was first described by Towbin et al. ***(1)***. Methanol is toxic and it can be omitted ***(38-40)***. Still, we use it to reduce swelling of the gel during transfer and to increase the binding of proteins to nitrocellulose ***(2, 38, 41)***. When working with high molecular weight proteins, elimination of the methanol results in a significant increase in protein transfer efficiency. Some recipes recommend the addition of low concentration of SDS to the buffer to help the transfer of high molecular weight proteins ***(36)*** and to improve the transfer of a variety of proteins ***(38)***. However SDS reduces the amount of protein bound to the membrane ***(2)*** and may adversely affect immunoreactivity by inhibiting renaturation of antigenic sites ***(42)***.

   For semidry blotting it is possible to use discontinuous buffer and/or "elution promoting" buffer on the gel side and a "retention promoting" buffer on the membrane side ***(43)***.

   To transfer hydrophobic proteins, such as membrane proteins, special buffers and transfer conditions are necessary ***(44)***.

3. Reagent grade methanol only is to be used because trace impurities in methanol can increase the conductivity of transfer buffer and decrease transfer efficiency.

4. Polyvinylidene difluoride (PVDF) may also be used ***(45, 46)***. Remember that unlike nitrocellulose, PVDF is a hydrophobic membrane and it must to be prewetted in methanol before use with aqueous solution. The buffer generally used to transfer proteins to PVDF is 10 m*M* 3-(cyclohexylamino)-1-propanesulfonic acid, 10% (v/v) methanol, pH 11 (47), although it is possible also the buffer described in Towbin et al. ***(1).***

   A noncostly modified western blot protocol, which uses metal nanoparticle-coated PVDF membranes, was recently described. It enables detection of small peptides (down to 2 kDa) with a sensitivity similar to that obtained for high molecular weight proteins. This modified PVDF prevents passage of small proteins through the membrane during electrotransfer and their loss during washing steps ***(48)***.

5. Electrotransfer is usually carried out immediately after the electrophoretic run from unstained gels. However, transfer of proteins from polyacrylamide gels after Coomassie blue or silver staining, has also been reported ***(49-51)***. Proteins can also be transferred for immunodetection from gels previously stained in a reverse (negative) way, e.g., with imidazole-zinc

salts ***(52).*** In these procedures, immunoreactivity pattern on the membrane, and total protein pattern can be obtained from the same gel from which spots have been transblotted, facilitating matching even with not easily reproducible 2-D separations.

6. Polyacrilamide IEF gels should be preequilibrated with transfer buffer containing 1% SDS and 20% glycerol, instead of methanol (to prevent swelling), since the focused proteins are at their isoelectric point and do not transfer well without equilibration. If it is desired to maintain the proteins in their native conformation, the SDS may be omitted, but the pH of the transfer buffer should be increase to pH 8.8 and the transfer may be less efficient ***(53)***.
7. The transfer efficiency is adversely affected by high molecular weight and basic pI of proteins. Therefore, while attempting to transfer slow proteins it may happen that fast proteins cross the nitrocellulose membrane and are lost. In these cases, one can use two stacked membranes, or membranes with smaller pore diameter which would also prevent loss of small polypeptides during membrane manipulation ***(54, 55)***. *See* **ref**. ***(56)*** for information on blotting on various membranes.

   Some low molecular weight, basic proteins, such as histones, lysozymes, cytochromes and so forth., do not transfer well because they may be near their pI in currently used buffers, as SDS is lost during the transfer in methanol. Transfer of these proteins can be improved, without impairing transfer of other proteins, by introducing a more basic transfer buffer and/or omitting the equilibration (**Subheading 3.1**, **step 3**) ***(57)***. Alternative buffers have also been proposed (e.g., ***(58)***).
8. When the transfer is conducted at high voltage it is necessary to refrigerate the transfer tank with a thermostatic circulator. If possible, avoid transfer in the cold room.
9. If it is only desired to check the immunoreactivity of an antibody towards a mixture of antigens, without attributing it to a particular protein, the antigen mixture (2–5 μl) can be spotted directly on the membrane. This technique, known as dot blotting ***(59)*** is useful for fast screenings of many antibodies simultaneously, for example in production of monoclonal antibodies.
10. In SDS gels, multicolored proteins can be used to provide a visual display of marker proteins on the transfer membrane. Various companies sell precolored standards and there is in the literature ***(60)*** a procedure for generating multicolored molecular weight proteins using a variety of Remazol-reactive textile dyes.
11. Chemical staining of proteins patterns transblotted onto the nitrocellulose or other membrane plays an important role in 2-D immunoaffinity identification, since it provides "landmark" spots to match immunoreactivity patterns to silver-staining patterns (cf. **Subheading 3.5** and **Note 18**). Several staining

procedures can be chosen. This step is usually carried out before the incubation of transblotted membranes with antibodies, employing dyes (e.g. Ponceau S, Fast Green, Amido black) or metal-chelates, which do not interfere with protein immunoreactivity ***(54, 61-64).*** Staining with substances such as Ponceau S, Fast Green, and metal-chelates is reversible, eliminating interference in the immunoreactivity pattern obtained with chromogenic substrates, but it is not very sensitive. A dye-based staining method, using Direct Blue 71 was recently developed. It is reversible, compatible with immunodetection and with sensitivity tenfold higher than Ponceau S ***(65)***. Permanent staining can also be used if the immunoreactivity pattern is collected from ECL-impressed films, but the stain must not interfere with immunoreaction.

Fluorescent dyes were also recently introduced for membrane staining. For example, SYPRO Ruby protein blot stain is a new, luminescent metal chelate stain composed of ruthenium in an organic complex that interacts no covalently with proteins. This stain is more sensitive than Ponceau, Coomassie Blue, Amido Black or India Ink and nearly as sensitive as colloidal gold stain procedures. This fluorescent stain is fully compatible with immunoblotting ***(66).***

When radioactive labelling is possible, most accurate total protein patterns can be collected from transblotted membranes by phosphor-imaging. The two images have the same dimensions so that general alignment and recognition of immunoreactive spots in the total protein pattern can be easily achieved. However the requirement of radioactive proteins is a huge limitation.

Perfect alignment of immunoreactive spots to total protein pattern can be obtained, at least with PVDF membranes, by the conjunction of colloidal gold staining for total protein detection and ECL for immunoreactivity on the same membrane ***(67).*** This procedure produce an ECL-impressed film with low exposure allowing the detection of immunoreactive spots and a ECL-impressed film with a strong exposure that produces a background pattern. The final result is a single image where immunoreactive spots appears as dark black spots and general protein pattern appears as light grey spots.

Colloidal gold ***(68, 69)*** and India Ink staining ***(70)*** can be applied also after immunodetection. The latter approach is possible if membrane saturation is achieved by Tween 20, omitting proteins ***(71, 72).***

Finally another method has been proposed by Zeindl-Eberhart ***(73)*** to localise easily antigen on 2-D gels. Proteins are transferred to PVDF membrane, immunostained with specific antibodies using Fast Red or 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a detection system, and then counterstained with Coomassie Brilliant Blue. The membrane appears with immunostained spots colored in red or black and the total protein pattern in blue. The blocking proteins are removed during the staining with Coomassie and so do not create a background staining.

In all these methods proteins are transferred onto one membrane and both total protein staining and immunostaining are performed on the same membrane. “Double replica” blotting methods were also developed, in order to obtain a membrane with all the proteins stained and that is an almost identical copy of the immunostained one. The first of its kind was described by Johansson ***(74)*** who found that by changing the direction of the blotting current the proteins could be transferred simultaneously from one gel onto two membranes, on either sides of the gel. A second method described by Neumann and Mullner ***(75)*** combines the usual electroblotting procedure with the generation of a “contact copy” from a gel. Both systems enable one membrane to be immunostained while the second membrane is stained by highly sensitive total protein staining methods. Protein identification is then carried out by comparing the signals of both matrices.

Similarly, a fast and simple method to produce print-quality like Ponceau replicas from blots was recently described ***(76).*** The positive replicas are the same size as blots and can be stored without loss of intensity. This makes them useful for localizing immunoreactive spots in complex 2-D electrophoretograms.

12. For a membrane from an one-dimensional SDS-gel or IEF gel we use 3–5 ml of solution according the dimension of the membrane in each washing and incubation step. For a 16 × 18 -cm membrane, we use 50 ml of solution. In general, volumes can be proportionally adjusted to other membrane dimension. It is important that the membrane is entirely soaked in solution during washing and incubation step.
13. Our blocking procedure is suitable for routine use. However, special conditions and reagents are required for immunoblotting with some antibodies, such as anti-phosphotyrosine antibodies ***(22, 77, 78).*** Information on different blocking conditions can be found in references ***(58, 79-81).*** Chemicon have developed a new blocking agent, composed of nonanimal proteins, that ensure uniform blocking, without nonspecific-binding, eliminating all cross-reactivity with animal source antigens, primary and secondary antibodies. Blocking with a nonionic detergent such as Tween 20, without added protein has also been used with the advantage that after immunodetection the blot can be stained for total protein pattern ***(71, 82, 83)*** (*see* **Note 10**). On the other hand, it has been found that blocking with detergent alone may cause loss of transblotted proteins ***(83, 84).*** Using PVDF membrane it is possible to employ a nonblock technique: this method incorporates 3 cycles of methanol-water hydration of the membrane, allowing multiple erasure and probing of the same blot with little or no loss of signal ***(85).***

   A modified Western Blotting protocol has been developed to increase the binding specificity of antigens and antibodies and clear the background.

The method is based on intermittent microwave irradiation of the blotting membrane in the immunoblotting step, using 5% skim milk as the diluting buffer ***(86)***. A simple method to improve western blotting and reduce background has been developed by Wu et al. ***(87)***, that consists in few modifications to the washing steps and buffer conditions.

14. Fixation of proteins to the membrane can be used to prevent their elution during washing and incubation steps. Some methods have been tried ***(42, 88)***, but the epitopes are sensitive to this treatment and may no longer be detectable by antibody. Another method is to fix the antibody-antigen complexes to the nitrocellulose membrane with glutaraldehyde, after they had been formed ***(89)***.
15. Optimal dilution of the primary and secondary antibody should be determined by immunoblotting of one-dimensional gels or dot blot analysis can be used. A technique called line blot assay has been developed to assess the optimal dilution of primary and secondary antibodies simultaneously ***(90)***. Working solutions of antibodies can be stored at −20°C and used several times ***(91)***.
16. Secondary antibodies often give problems of cross-reactivity, especially in the analysis of samples containing antibodies (such as immunoprecipitates, immune tissue, plasma, etc.), even when antibodies from different species are used. Langstein and Schwarz proposed a method to avoid this problem, that consist in preconjugating the primary and secondary antibody before incubation. In this way, signals from secondary antibodies are completely quenched ***(92)***. An other solution to this problem is “double-blotting”. After the membrane has been incubated with the primary antibody, it is blotted a second time under acidic conditions. Antigen and interfering proteins remain bound to the first membrane, and the primary antibodies were transferred to the second one, that can be probed with secondary antibodies, without nonspecific binding ***(93)***.

    Alternatively, HRP-conjugated protein A and protein G, which detect intact antibody molecules almost exclusively, can be used to obtain clean and specific western blot signals of target proteins ***(94)***.
17. A novel cross-linked enzyme-antibody conjugates has been developed for Western blot and ELISA ***(95)***. It was prepared by cross linking of HRP to a high molecular weight polyglutamic acid polymer followed by the cross linking of a limiting amount of antibody to yield a complex with a detection signal 100-fold greater than that of 1:1 enzymes conjugates usually prepared by cross-linking.
18. The secondary antibody we use is labelled with peroxidase. The major drawback of this approach is that the range of protein loading that gives a linear relationship between the amount of target protein and the signal

is quite limited. Considerable advantage for quantitative analysis can be gained by the use of a secondary antibody coupled to fluorophores that allow quantification of fluorescent signal, e.g. by means of a phosphor imager device. This approach theoretically gives a linear signal through a broad range of protein loading ***(96, 97)***.

19. ECL detects horseradish peroxidase-conjugated antibodies through oxidation of luminol, in the presence of hydrogen peroxide and a phenolic enhancer under alkaline conditions. ECL reagents are capable of detecting 1–10 pg of protein antigen. Alternative enhancers that extend the duration of light emission and enable such system is ECL plus (Amersham Biosciences) ***(98).*** These systems are suitable for use of charge-coupled device (CCD) camera that require longer exposure times for good quantification of immunoreaction.
20. In case chemiluminescence is too strong or background is too high, one can switch to detection with a chromogenic substrate.

    We use 4-chloro1-naphthol ***(99)*** as chromogenic substrate, according to the following protocol:
    (a) After ECL detection (or after step 7 of **Subheading 3.3.1**), wash the membrane briefly with 0.05 M Tris-HCl, pH 6.8.
    (b) Soak it in developing solution (20 ml 0.05 M Tris-HCl pH 6.8, 7 µl H_2O_2 30% (v/v), 5 ml 4-chloro1-naphthol 0.3% (w/v) in methanol) until the color appears. Stop the reaction with washes in distilled water.
    (c) Air dry the membrane and photograph it as soon as possible, because the color fades with time.
21. Chemiluminescent probes enable highly sensitive quantitative analysis of proteins blotted from electrophoretic gels onto a supporting matrix. For a quantitative comparison, it is important to be able to correct for the variations introduced by variables such as antibody titre, temperature, substrate etc.. Comparison of separate blots completed on different days requires a chemiluminescent standard. The situation is more complex with 2 D gels, where only one sample per gel/blot is used. A method has been published for preparing chemiluminescent standard for quantitative comparison of 2D western blot ***(100)***.
22. In detection of Western blots of total cell lysates there is the problem of nonspecific background chemiluminescence, due to nonspecific binding of primary and secondary antibody. A noncommercial ECL solution that provides strong signals and low background for western blot detection was recently developed ***(101)***. This alternative solution does not contain chemicals which harm the blots, making it possible redetect them after stripping up to five times. Due to the strong signals and low background of this ECL-solution, the primary and secondary antibodies used for detection can also be used at lower concentrations.

23. It is also possible to perform stripping with kits as the CHEMICON Re-Blot™ western blot recycling kit. Stripping of antibodies also elutes antigens from the membrane and signal intensity decreases in successive cycles. It is important therefore to remember that stripping should be used only for qualitative purpose.

    As alternatives to stripping, one can use: (a) different chromogenic substrates for peroxidase at each cycle (rainbow blotting, ***(91)***), (b) ECL and inactivating peroxidase after each cycle ***(91),*** (c) different labels and detection methods at each cycle ***(102).***
24. To perform the matching process we use the software Melanie 4 (Gene Bio).
25. Matching can also be done by simple eye inspection directly on nitrocellulose and ECL film when the sample contains relatively few spots, all of them detectable by chemical staining of nitrocellulose. In the majority of cases samples are very complex and many low abundance proteins occur. In these cases matching by computer is mandatory, in order to identify immunoreactive spots in silver-stained patterns. The following manual procedure is suggested:
    (a) Match the exposed film with nitrocellulose membrane, aligning the upper left corner and the two corresponding borders and placing the cut lower right corner in the same orientation for both.
    (b) Using a waterproof pen, mark the other two borders of the nitrocellulose on the film and transfer the chemically stained spot present on nitrocellulose on the ECL film in order to use them as landmarks for the next matching with the silver nitrate stained gel.
    (c) Nitrocellulose membrane and film maintain the initial size, but the size of the gel increases after silver-staining. Size equalization can be obtained by photographic or photocopy procedures.
    (d) On a transilluminator match all the landmarks with the corresponding spots on the silver nitrate stained gel to identify the immunoreactive spots.

    When the area of the membrane containing the protein of interest is known, another procedure, described by Lindahl ***(103),*** can be used. Only a limited area of the nitrocellulose containing the proteins is cut out and incubated with antibodies. The rest of the membrane is stained with one of the methods described in **Note 10** (a method not compatible with immunodetection, but much more sensitive than Ponceau S, can be used). Thus the protein spots could be located in the 2-DE pattern, matched with a corresponding silver stained gel and translated into protein pattern. This method allows also a considerable saving of antibodies.
26. We use a computing densitometer 300 S from Molecular Dynamics with a resolution of 4000 × 5000 pixels, 12 bits/pixel, which generate 40 megabytes images on 16 bits.

27. The silver stained image used for matching can be taken from your archive of files or from images available in Internet, provided that the identical electrophoretic procedures have been applied. The possibility to match images deriving from different 2-D electrophoretic procedures has been studied by Lemkin P. ***(104).***
28. This step may be difficult if the "landmark" spots stained by Red Ponceau on the nitrocellulose membrane are few. To aid recognition of the spots chosen as landmarks on the silver stained gel, we suggest also staining with silver the gel from which the proteins are transferred, in which the amount of proteins loaded must be twice that used for a silver-stained gel. Most spots will be still visible on the transferred gel and can be used for a first matching with the membrane. Using the gel from which the membrane was obtained, the landmarks can be localized correctly. The landmarks are then easily transferred to the silver stained gel by computer matching.
29. The mapping of immunoreactive spots on 2-D silver-stained reference images can be improved if the images of the Ponceau- and immunostained membranes are acquired with the same system, enabling detection of total protein pattern directly on the membrane where the immunoreaction takes place. In commercially available gel and blot image acquisition systems, images generated in different ways can be readily acquired on the same instrument.

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High-Efficiency Blotting of High-Molecular Weight Proteins

Marion L. Greaser and Darl R. Swartz

1. Introduction

The technique of protein electrophoresis in the presence of sodium dodecyl sulfate has now been conducted for more than 40 years. Early work employed single layer gel systems ***(1,2).*** It was subsequently shown that a stacking/discontinous gel system gave superior results, and the method of Laemmli ***(3)*** has been widely adopted. Numerous modifications of this original system have developed through the years, although many investigators continue to use the Laemmli procedures as originally described.

Adaptations to the Laemmli system have been made in our laboratory specifically to address difficulties in resolving proteins in muscle tissue. Changes in acrylamide to bisacrylamide ratio to 200:1 (vs 37.5 to 1 in the Laemmli system) and increasing the buffer concentration in the separating gel were found to aid in resolution of proteins in the 75 to 10 kDa range ***(4).*** Solubilization of the tough, fibrous heart and skeletal muscle was improved by using an "industrial strength" sample buffer containing non-ionic denaturants (urea and thiourea) in addition to SDS plus a sulfhydryl reducing agent ***(5).*** A further adaptation has been made to increase the migration of very high molecular weight protein into a polyacrylamide gel. Muscle contains two extremely large proteins, titin (Mr ~ 3500K Da) and nebulin (Mr 900 KDa), that barely enter a typical Laemmli gel because they do not stack properly in a conventional bisacrylamide cross-linked stacking gel. This adaptation involves the use of DATD (N, N -diallyltartardiamide) as the stacking gel cross-linking agent ***(6).***

A further modification that has ramifications in regard to blotting high molecular weight proteins is the inclusion of a reducing agent in the upper reservoir buffer during electrophoresis and in the subsequent protein transfer

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to the membrane ***(6).*** Conditions during electrophoresis and blotting favor formation of inter and intramolecular disulfide crosslinks. Although the electrophoresis and blotting systems described here were designed primarily for work with muscle, the methods work equally well with other tissues. A particular advantage is the wide molecular weight range achieved without the use of a gradient gel system **(Fig. 1).**

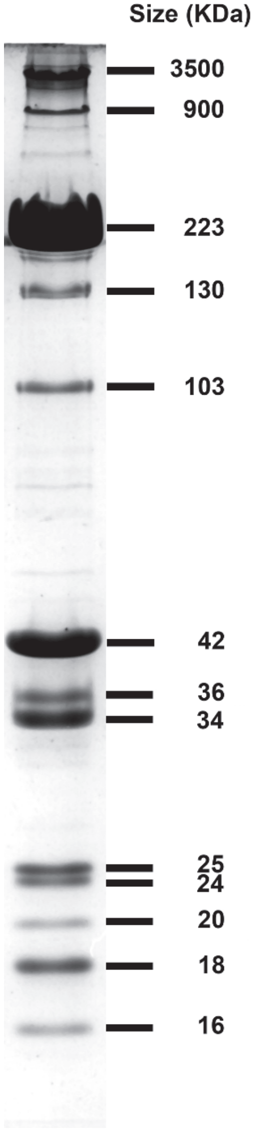

Fig. 1. Rabbit skeletal muscle myofibril proteins on a SDS 10% polyacrylamide gel (200:1 ratio of acrylamide to bisacrylamide separating gel; 3% acrylamide with DATD cross-linker in the stacking gel), separating gel pH 9.3. Only the separating gel is shown. Proteins with sizes from 15 to 3500 KDa can be resolved on gels with a single concentration of acrylamide.

2. Materials

2.1. Apparatus

1. Mighty Small II (SE 250 or 260) Slab Gel Unit (Hoefer – http://www.hoeferinc.com/ or a similar commercial gel unit).
2. A constant current power supply – available from above company as well as other sources.
3. TE62 Tank Blotting Unit (Hoefer).

2.2. Stock Solutions

1. Resolving gel acrylamide; 30% T – 0.5% C. Weight 29.85 g of acrylamide and 0.15 g Bis-acrylamide into a beaker, add about 50 ml of water, stir till dissolved, dilute to 100 ml. Filter through a 0.45 micron filter (such as a Millex-HA, Millipore Corporation, Bedford, MA 01730). Store in a brown bottle in the cold room (4°C). Danger! Avoid skin contact (*see* **Note 1**).
2. Stacking gel acrylamide; 10% T – 15% C. Weigh 4.25 g acrylamide and 0.75 g of DATD into a beaker, add 25 ml of water, stir till dissolved, dilute to 50 ml. Filter as above, store in brown bottle at 4°C, avoid skin contact **(***see* **Note 2)**.
3. 3 *M* Tris-HCl, pH 9.3; Dissolve 32.2 g Trizma base (desiccated) and 5.4 g Tris-HCl (desiccated) in water, dilute to 100 ml. Store at room temperature (shelf life of about 1 month). If desiccated reagents are used, no pH adjustment is necessary. Alternative solution; 3M Tris-HCl, pH 8.8; use 26.16 g Trizma base and 13.26 g Tris-HCl per 100 ml **(***see* **Note 3)**.
4. 0.5 *M* Tris-HCl, pH 6.8; Dissolve 0.075 g Trizma base (desiccated) and 3.85 g Tris-HCl (desiccated) in water, dilute to 50 ml. Store in the cold.
5. 10% (w/v) SDS; Dissolve, filter through 0.45 micron filter. Store at room temperature.
6. Reservoir buffer concentrate (5X); 0.25 *M* Tris base, 1.92 M glycine, 0.5% SDS. Store at room temperature.
7. Ammonium persulfate; 100 mg/ml solution in water; store frozen in 0.5 ml aliquots (stable indefinitely at –20°C) **(***see* **Note 4)**.
8. Sample buffer A; 8 *M* urea, 2 M thiourea, 0.05 *M* Tris-HCl (pH 6.8), 75 m*M* DTT, 3% SDS, 0.05% bromophenol blue ***(5)*** **(***see* **Note 5)**.

Recipe for 100 ml of sample buffer:

a. Weigh 48g urea (ACS grade) and 15.2g thiourea into a clean 150ml glass beaker, add a stir bar, and 40ml of water (careful! Do not add too much water; the urea plus thiourea takes up over half the final volume). Use latex gloves to avoid contamination of the sample buffer with skin proteins. Also avoid skin contact with the thiourea since it is a suspected carcinogen.
b. Stir gently on a hot plate until the solution is at room temperature and all the solids are dissolved (watch carefully, avoid temperatures above 40°C - heating urea speeds the formation of cyanate).
c. Add 10 g of a mixed bed resin (Biorad AG 501-X8 or Thermo Fisher Rexyn 300), stir mixture at room temperature for 15 min. If you have a conductivity meter the conductivity should be less than 5 µmhos **(***see* **Note 6)**.

d. Filter the mixture through filter paper into a 100ml graduated cylinder. The volume should be 80–90 ml. Carefully rinse the resin with one or two 5 ml aliquots of de-ionized water. Transfer the filtered solution back to a clean 150 ml beaker.
e. Weigh 0.605 g Trizma base, and 3 g SDS; add to the urea-thiourea solution and stir till dissolved. Adjust pH to 7.5 carefully; add 12M HCl 100 µl at a time (300 to 400 µl required). Add 1.155g of solid DTT and stir till dissolved.
f. Continue adjusting pH down to 6.8 using 20 µl aliquots of 2 *M* HCl (the buffer capacity rapidly declines as the pH is lowered- use care not to overshoot!). However if you mistakenly go past 6.8, adjust back up with 2 *M* Tris base. Add 2–4 mg bromophenol blue, stir till dissolved.
g. Transfer solution back to the 100 ml graduated cylinder, add water to 100ml, mix. Filter through a Millex HA 0.45 or 0.22 µm filter (Millipore) to remove any fine particulate material. Transfer filtered sample buffer to 1ml or 0.5ml Microfuge vials; store at (−) 20°C, thaw amount needed just before use.

9. Sample buffer B; 0.05 *M* Tris-HCl (pH 6.8), 75 m*M* DTT, 3% SDS, 10% glycerol, 0.1% bromophenol blue.
10. 50% v/v glycerol.
11. Staining solution; Weigh 0.5 g of Coomassie brilliant blue R-250 and dissolve in 500 ml of methanol, add 400 ml H_2O, and 100 ml acetic acid. Store at room temperature. Use once and discard. The stock solution should be discarded if a scum appears on top of the solution.
12. Transfer buffer: 20 m*M* Tris base, 150 m*M* glycine, 20% v/v methanol ***(7, 8)*** (or 10 m*M* CAPS pH 11, 10% methanol) ***(9)***. For high molecular weight proteins, add SDS and 2-mercaptoethanol to 0.1% and 10 m*M* respectively to the transfer buffer.

3. Methods

3.1. Gel Preparation

1. Resolving gel for four Mighty Small gels, 0.75 mm thick (12% acrylamide); Into a 125 ml filtering flask, add the following: 9.6 ml of 30% Resolving gel acrylamide, 6.0 ml of 3 *M* Tris, pH 9.3 or 8.8 (see above), 0.24 ml of 10% SDS, 0.15 ml of 100 mg/ml ammonium persulfate, 3.20 ml of water, 4.80 ml of 50% v/v glycerol. (Total acrylamide concentration can be varied, but sum of acrylamide and water volumes should be 14.4 ml). Swirl to mix thoroughly, attach to a water aspirator to degas the solution (1 min) **(*see* Note 7).**
2. Add 0.015 ml of TEMED, swirl to mix, immediately pour solution into the previously assembled slab gel casting unit (use clear plastic spacers between the gel sandwiches rather than wax paper to prevent the gel sandwiches from sticking together) and cover each gel with 0.075 ml of water-saturated isobutanol. Allow to polymerize. The optimal time is 10 to 20 min. If the gel sets more quickly, decrease the amount of ammonium persulfate; if the gel takes longer, increase the ammonium persulfate. There is considerable batch to batch variation in the acrylamide and ammonium persulfate so the levels need to be readjusted with your own reagents. Also the degree of degassing affects the polymerization rate– dissolved oxygen retards polymerization. The acrylamide is gelled when a clear line can be observed

1–2 mm below the isobutanol or the remaining acrylamide mixture has polymerized in the flask.

3. Rinse off isobutanol and unpolymerized upper layer with water. Disassemble the casting unit (use gloves to handle the plates). The gel plus glass plates can be used immediately or stored for several days at 4°C in a closed plastic bag (to avoid evaporation). For immediate use, place the rinsed gel sandwich(s) in the Mighty Small unit. If only one gel is used, it is best to place a blank plate on the opposite side to prevent a direct current connection to the lower chamber.
4. Stacking gel for two Mighty Small gels (3% acrylamide); Into a 50 ml filtering flask, add the following: 0.750 ml of 10% Stacking gel acrylamide, 0.625 ml of 0.5 *M* Tris (pH 6.8), 0.025 ml of 10% SDS, 0.015 ml of 100 mg/ml ammonium persulfate, 0.572 ml of water, 0.500 ml of 50% v/v glycerol, a couple drops of bromophenol blue. Swirl to mix, degas 1 min (optional), add 0.012 ml TEMED, swirl to mix, immediately transfer to the top of the slab gel. Insert the sample combs, avoid trapping air bubbles. Allow to polymerize (*see* **Note 8**).

3.2. Sample Preparation

1. Whole tissue (fresh or frozen); Portions (25 to 35 mg) of whole tissue are weighed into a preweighed 5 ml glass homogenizing tube. Add 140 mg (or µl) of sample buffer A per mg tissue, homogenize to disperse tissue chunks, transfer sample to a Microfuge tube, and then heat in a block heater set at 100°C for 3 min. (Avoid heating proteins in sample buffer for more than 5 min at 100°C; the urea will form cyanate which reacts with proteins and alters migration rates.) Cool to room temperature. Samples should be prepared immediately before application to the gels. If specimens must be stored before analysis, it is better to keep the *tissue* at −20°C or −80°C rather than storing the proteins in sample buffer. Samples in sample buffer can be stored at −80°C; it is not necessary or advisable to heat them again other than for thawing.
2. Tissue homogenates; Mix tissue suspensions (1–5 mg/ml) with an equal volume of sample buffer A in a Microfuge tube. Heat 3 min in a block heater or boiling water bath (*see* **Note 9**).
3. Soluble proteins; Pipette 0.05 ml of protein solution (1 to 5 mg/ml) into a Microfuge tube. Add 0.05 ml of sample buffer A or B, mix. Heat for 3 min in a block heater or boiling water bath.

With all kinds of samples, avoid protein concentrations exceeding 5mg/ml.

3.3. Sample Loading and Electrophoretic Separation

After polymerization, fill the upper and lower reservoirs with buffer (0.05 M Tris, 0.384 M glycine, 0.1% SDS). Add 10 mM 2-mercaptoethanol to the upper reservoir solution (stock solution is 14.3 molar). Gently remove the gel combs after upper reservoir is filled. Load sample wells using either a thin tipped Pipetman or 10 µl glass syringe. Best resolution is achieved if the sample is deposited in a narrow zone at the bottom of the well. Apply constant current (20 mA/per gel) until dye front moves to the bottom of the separating gel (run time usually about 1 h).

3.4. Western blotting

After electrophoresis, the gel is removed from the plates, submerged in transfer buffer for 15 min with gently shaking. The gel is next placed over either a sheet of nitrocellulose or PVDF (polyvinylidene difluoride) and moved to the transfer tank. Transfers were completed using a current of 0.5 A for 30 to 60 min with circulated water cooling. The CHAPS transfer buffer with added 0.1% SDS and 10 m*M* 2-mercaptoethanol works best for transfer of high molecular weight proteins. It is recommended that the gel be stained after the transfer to assess if protein remains in the gel. Usually all proteins are fully transferred except titin (*see* **Notes 10** and **11**).

4. Notes

1. Increasing the Tris concentration in the separating gel (compared to the Laemmli method) improves the resolution of lower molecular weight proteins.
2. Use of DATD instead of bisacrylamide as the cross-linker for the stacking gel was found to be important to get proper migration of the very high molecular weights into the separating gel. It appears that the stacking gel pore size in a conventional Laemmli type system retards titin and nebulin so these proteins do not stack properly. If the protein is lost from the "stack" during the stacking process, it is subjected to a much different electrophoretic conditions than those in the stack and thus lags behind. A simple test for the performance of the stacking gel is to stain the entire gel and look for proteins in the stacking gel. If it is working properly, there should no protein in the stacking gel.
3. Using desiccated and weighed Tris base and Tris-HCl to make the separating and stacking gel buffers is both convenient and eliminates buffer pH variations. Also many pH electrodes do not respond well to high concentrations of Tris, so pH adjustment becomes problematic. The pKa of Tris has a high temperature coefficient, and this further complicates use of a pH electrode. The pH of the resolving gel can be lowered to about 8.6 to increase the migration at the cost of a longer run time. The acrylamide percentage of the resolving gel may also be reduced to about 8% to improve migration. Combinations of lower pH and lower acrylamide may also be employed to optimize resolution of the high molecular weight size range of interest. For high resolution electrophoresis and blotting of very high MW proteins, an agarose gel system has been developed ***(11).***

4 Preparing a large batch of ammonium persulfate stock solution and storing aliquots frozen at –20°C saves time (compared to repeated weighings) and gives repeatable polymerization conditions each time gels are poured.

5. Solubilization of tough, fibrous tissues like cardiac or skeletal muscle is facilitated by using an SDS buffer containing urea and thiourea. Whole tissue chunks need to be mechanically dispersed using some type of

homogenizer to dissolve all the protein. The Dounce type homogenizer seems to work best since it avoids excessive foaming that can occur with spinning blade type homogenizers. Non-urea-thiourea sample buffers often contain tissue fragments that must be removed by 2–5 min centrifugation in a Microfuge at 14,000 × g. If these fragments are not removed, they lead to protein streaking on gels. Use of the industrial strength sample buffer reduces this problem and samples usually do not require centrifugation. DTT is the preferred reducing agent in the sample buffer as it is much better at reducing intra/inter molecular disulfides than 2-mecaptoethanol and is not volatile.

6. Treatment of the urea and thiourea solutions with a mixed bed ion exchange resin is necessary to remove ionic contaminants from these reagent grade chemicals.
7. Inclusion of glycerol in the separating and stacking gel layers increases the viscosity and results in sharper protein bands. Thin (0.75 mm) and small for-

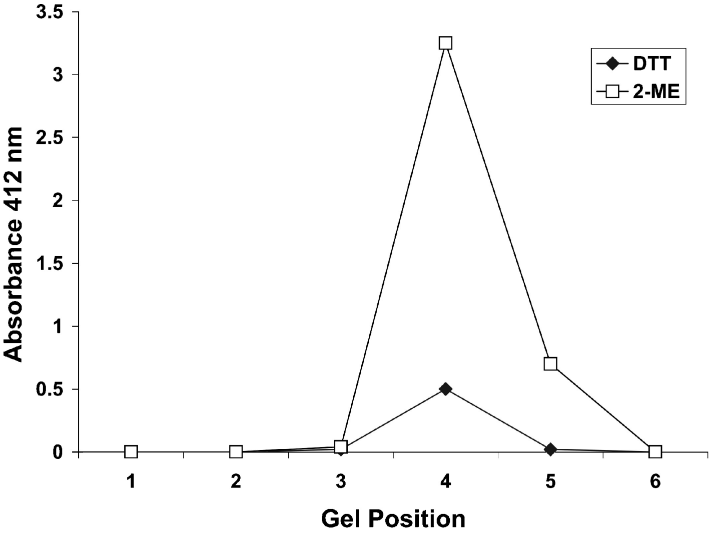

Fig. 2. Reducing agent migration through a polyacrylamide gel. Reducing agents, either 37.5 m*M* dithiothreitol or 0.35 *M* 2-mercaptoethanol, were added along with bromophenol blue tracking dye to the sample wells (gel position 1). Current was applied until the tracking dye migrated half way down the gel (gel position 4). The presence of reducing agent was determined by cutting out gel slices and incubating them with Ellman's reagent *(7)* to detect free sulfhydryl groups (re-drawn from *[6]*). Both reducing agents migrated at the same position as the tracking dye. Inclusion of a reducing agent in the upper reservoir buffer during electrophoresis results in the proteins being continuously bathed in reductant throughout the electrophoresis run.

mat gels (8 × 10 cm) are preferred as their run time is short and transfer of proteins is more complete.

8. We add a couple drops of 0.05% bromophenol blue to the stacking gel mixture before polymerization. This makes the stacking gel slightly blue and aids in identifying the well positions for subsequent sample application.
9. Samples with high KCl concentrations should be diluted with water or dialyzed to remove the KCl as the potassium forms a precipitate with SDS. Alternatively, protein samples in high KCl can be mixed with 4 volumes of methanol (−20°C), microfuged for 1 min, the solution discarded, and the methanol removed by brief vacuum treatment. The pellets can then be dissolved in the urea-thiourea sample buffer diluted 1:1 with water. The methanol precipitation procedure can also be employed to concentrate proteins that are too dilute.
10. Addition of mercaptoethanol to the upper reservoir buffer has been found to be important to get migration of the very large molecular weight proteins into the separating gel. The reducing agents, such as 2-mercaptoethanol or dithiothreitol typically used in sample buffers, are charged at pH values between 8 and 10 and move though the gel with the tracking dye front ***(6)*** (Fig. 2). The poor protein migration (smearing, retardation, additional bands of still higher molecular weight) of very large proteins when the upper reservoir does not include a sulfhydryl reducer suggests that intermolecular and intramolecular disulfide bonds are being formed during the electrophoresis run.
11. The disulfide bond formation of large proteins during electrophoresis also retards their migration out of the gel onto blots during transfer. Thus inclusion of 2-mercaptoethanol in the transfer buffer improves efficiency of transfer of high molecular weight proteins. Semidry blotting equipment can substituted for the tank system.

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Alkaline Phosphatase Labeling of IgG Antibody

G. Brian Wisdom

1. Introduction

Alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa has proven, over several decades, to be a very valuable label for antibodies. It is stable, has a moderate size (140 kDa), a high turnover number, and can be assayed using a variety of different substrates which are measured by changes in absorbance, fluorescence, or luminescence. The most common method of labeling immunoglobulin G (IgG) antibody with this enzyme uses the homobifunctional reagent glutaraldehyde.

The chemistry of glutaraldehyde is complex. It reacts with the amino group and, to a lesser extent, the thiol group of proteins and when two proteins are mixed in its presence, stable conjugates are produced without the formation of Schiff bases. Self-coupling can be a problem unless the proteins are at appropriate relative concentrations. In the method described ***(1)*** there is usually little self-coupling of the enzyme or the IgG antibody, however, the size of the conjugate is large ($>10^6$ Da) as several molecules of each component are linked. This is the most simple labeling procedure to carry out and, although the yields of enzyme activity and immunoreactivity are small, the conjugates obtained are stable and practical reagents.

Alkaline phosphatase may also be coupled using heterobifunctional reagents containing the *N*-hydroxysuccinimide and maleimide groups, for example, succinimidyl 4-(*N*-maleimidyl)-cyclohexane-1-carboxylate. However, because the enzyme has no free thiol groups this approach is usually used for the labeling of $F(ab')_2$ fragments of IgG via their thiols ***(2)***.

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2. Materials

1. Alkaline phosphatase from bovine intestinal mucosa. 2000 U/mg (with 4-nitrophenyl phosphate as substrate). There are numerous commercial sources of labeling-grade enzyme; it is usually supplied at a concentration of 10 mg/mL in phosphate or triethanolamine buffer (this avoids interference with the conjugation). If ammonium sulfate, Tris or primary or secondary amine is present, it must be removed (*see* **Note 1**).
2. IgG antibody. This should be the pure IgG fraction or, better, affinity-purified antibody from serum or pure monoclonal antibody (*see* Chapters 179–190).
3. Phosphate-buffered saline (PBS): 20 m*M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* NaCl.
4. Glutaraldehyde.
5. 50 m*M* Tris-HCl buffer, pH 7.5, containing 1 m*M* $MgCl_2$, 0.02% NaN_3, and 2% bovine serum albumin (BSA).

3. Methods

1. Add 0.5 mg of IgG antibody in 100 µL of PBS to 1.5 mg of alkaline phosphatase.
2. Add 5% glutaraldehyde (about 10 µL) to give a final concentration of 0.2% (v/v) and stir the mixture for 2 h at room temperature.
3. Dilute the mixture to I mL with PBS and dialyze against PBS (2L) at 4°C overnight (*see* **Note 1**).
4. Dilute the solution to 10 mL with 50 m*M* Tris-HCl buffer, pH 7.5, containing 1 m*M* $MgCl_2$, 0.02% NaN_3, and 2% BSA, and store at 4°C.

4. Notes

1. Dialyses of small volumes can be conveniently done in narrow dialysis tubing by placing a short glass tube, sealed at both ends, in the tubing so that the space available to the sample is reduced. Transfer losses are minimised by carrying out the subsequent steps in the same dialysis bag. There are also various microdialysis systems available commercially such as the Slide-A-Lyzer units from Pierce Biotechnology (Rockford, IL).
2. The conjugates are stable for several years at 4°C as the NaN_3 inhibits microbial growth and the BSA minimizes denaturation and adsorption loses. The conjugates should not be frozen.
3. Purification of the conjugates is usually unnecessary; however, if there is evidence of free antibody it can be removed by size-exclusion chromatography in Sepharose CL-6B (GE Healthcare Bio-sciences, Uppsala, Sweden and Piscataway, NJ) or a similar gel with PBS as solvent.
4. The efficacy of the enzyme-labeled antibody can be tested by immobilizing the appropriate antigen on the wells of a microtiter plate or strip, incubating various dilutions of the conjugate for a few hours, washing the wells, adding substrate, and measuring the amount of product formed. This

approach may also be used for monitoring conjugate purification of chromatographic fractions.

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β-Galactosidase Labeling of IgG Antibody

G. Brian Wisdom

1. Introduction

The *Eschericia coli* β-galactosidase (EC 3.2.1.23) is a large enzyme (465 kDa) with a high turnover rate and wide specificity which allows it to be measured conveniently in several different ways. Unlike several other enzyme labels β-galactosidase is not found in mammalian tissues or fluids hence background contributions are negligible when this label is measured at a neutral pH. This can be advantageous as peroxidase activities (due to various heme proteins) and alkaline phosphatase are frequently present in these samples.

The heterobifunctional reagent, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), is of value when one of the components of a conjugate has no free thiol groups eg. IgG. In this method ***(1)*** the IgG antibody is first modified by allowing the *N*-hydroxysuccinimide ester group of the MBS to react with amino groups in the IgG; the β-galactosidase is then added and the maleimide groups on the modified IgG react with thiol groups in the enzyme to form thioether links. This procedure produces conjugates with molecular weights in the range $0.6\text{-}1 \times 10^6$.

$F(ab')_2$ fragments of IgG have been labeled via the β-galactosidase's amino groups with sulfo-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate ***(2)*** and there are various other heterobifunctional reagents that may be used for β-galactosidase labelling ***(3)***.

2. Materials

1. β -Galactosidase from *E. coli*, 600 U/mg or greater (with 2-nitrophenyl-β -galactopyranoside as substrate) (*see* **Note 1**).

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2. IgG antibody. This should be the pure IgG fraction or, better, affinity-purified antibody from an antiserum or pure monoclonal antibody.
3. 0.1 *M* Sodium phosphate buffer, pH 7.0, containing 50 mM NaCl
4. MBS.
5. Dioxan.
6. Sephadex G25 (GE Healthcare Bio-sciences, Uppsala, Sweden and Piscataway, NJ) or an equivalent gel.
7. 10 m*M* Sodium phosphate buffer, pH 7.0, containing 50 m*M* NaCl and 10 m*M* $MgCl_2$.
8. 2-Mercaptoethanol.
9. DEAE-Sepharose (GE Healthcare Bio-sciences) or an equivalent ion-exchange medium.
10. 10 m*M* Tris-HCl buffer, pH 7.0, containing 10 m*M* $MgCl_2$ and 10 m*M* 2-mercaptoethanol.
11. Item 10 containing 0.5 *M* NaCl.
12. Item 10 containing 3% bovine serum albumin (BSA) and 0.6% NaN_3.

3. Methods

1. Dissolve 1.5 mg of IgG in 1.5 mL of 0.1 *M* sodium phosphate buffer, pH 7.0, containing 50 m*M* NaCl.
2. Add 0.32 mg of MBS in 15 µL of dioxan, mix, and incubate for 1 h at 30°C.
3. Fractionate the mixture on a column of Sephadex G25 (approx. 0.9 × 20 cm) equilibrated with 10 m*M* sodium phosphate buffer, pH 7.0, containing 50 m*M* NaCl and 10 m*M* $MgCl_2$, and elute with the same buffer. Collect 0.5 mL fractions, measure the A_{280} and pool the fractions in the first peak (about 3 mL in volume).
4. Add 1.5 mg of enzyme, mix, and incubate for 1 h at 30°C.
5. Stop the reaction by adding 1 *M* 2-mercaptoethanol to give a final concentration of 10 m*M* (about 30 µL).
6. Fractionate the mixture on a column of DEAE-Sepharose (approx. 0.9 × 15 cm) equilibrated with 10 m*M* Tris-HCl buffer, pH 7.0, containing 10 m*M* $MgCl_2$ and 10 m*M* 2-mercaptoethanol; wash the column with this buffer (50 mL) followed by the buffer containing 0.5 *M* NaCl. (50 mL). Collect 3 mL fractions in tubes with 0.1 mL of Tris buffer containing 3% BSA and 0.6% NaN_3. Pool the major peak (this is eluted with 0.5 M NaCl), and store at 4°C (*see* **Notes 2** and **3**).

4. Notes

1. The thiol groups of β-galactosidase may become oxidized during storage thus diminishing the efficacy of the labeling. It is relatively easy to measure these groups using 5,5 -dithiobis(2-nitrobenzoic acid) (*see* Chapter 93); about 10 thiol groups per enzyme molecule allow the preparation of satisfactory conjugates.

2. The conjugates are stable for a year at 4°C as the NaN_3 inhibits microbial growth and the BSA minimizes denaturation and adsorption losses.
3. The activity of the conjugate can be checked by the method described in **Note 4** of Chapter 64.

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Horseradish Peroxidase Labeling of IgG Antibody

G. Brian Wisdom

1. Introduction

Horseradish peroxidase (HRP; EC 1.11.1.7) is probably the most widely used enzyme label. This protein is relatively small (44 kDa), stable, and has a broad specificity which allows it to be measured by absorption, fluorescence or luminescence. Some of its products are intensely coloured, which makes this enzyme suitable for immunocytochemical or immunoblotting applications.

The most popular method ***(1)*** for labeling IgG antibody molecules with HRP exploits the glycoprotein nature of the enzyme. The saccharide residues are oxidized with sodium periodate to produce aldehyde groups that can react with the amino groups of the IgG molecule, and the Schiff bases formed are then reduced to give a stable conjugate of high molecular weight ($0.5–1.0 \times 10^6$). The enzyme has few free amino groups so self-coupling is not a significant problem.

Other methods have been described for using this enzyme as a horseradish peroxidase as a label. IgG may be labeled with HRP using glutaraldehyde in a two-step proecure ***(2)*** and, when partially reduced, IgG has been labelled using the heterobifunctional succinimidyl 4-(*N*-maleimidyl)-cyclohexane-1-carboxylate ***(3)***.

2. Materials

1. Horseradish peroxidase, 1000 U/mg or greater (with 2,2´-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] as substrate) (*see* **Note 1**).
2. IgG antibody. This should be the pure IgG fraction or, better, affinity purified antibody from an antiserum or pure monoclonal antibody (*see* Chapters 179–189).
3. 0.1 *M* Sodium periodate, freshly prepared.
4. 1 m*M* Sodium acetate buffer, pH 4.4.
5. 10 m*M* Sodium carbonate buffer, pH 9.5.

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6. 0.2 *M* Sodium carbonate buffer, pH 9.5.
7. Sodium borohydride, 4 mg/mL (freshly prepared).
8. Sepharose CL-6B (GE Healthcare Bio-sciences, Uppsala, Sweden and Piscataway, NJ) or a similar gel.
9. PBS: 20 mM sodium phosphate buffer, pH 7.2, containing 0.15 *M* NaCl.
10. Bovine serum albumin (BSA).

3. Method

1. Dissolve 2 mg of peroxidase in 500 µL of water.
2. Add 100 µL of freshly prepared 0.1 *M* sodium periodate, and stir the solution for 20 min at room temperature. (The color changes from orange to green).
3. Dialyze the modified enzyme against 1 m*M* sodium acetate buffer, pH 4.4 (2 L) overnight at 4°C (*see* **Note 2**).
4. Dissolve 4 mg of IgG in 500 µL of 10 m*M* sodium carbonate buffer, pH 9.5.
5. Adjust the pH of the dialyzed enzyme solution to 9.0–9.5 by adding 10 µL of 0.2 *M* sodium carbonate buffer, pH 9.5, and immediately add the IgG solution. Stir the mixture for 2 h at room temperature.
6. Add 50 µL of freshly prepared sodium borohydride solution (4 mg/mL), and stir the mixture occasionally over a period of 2 h at 4°C.
7. Fractionate the mixture by size-exclusion chromatography in a column (approx. 1.5 × 85 cm) of Sepharose CL-6B in PBS. Determine the A_{280} and A_{403} (*see* **Note 3**)
8. Pool the fractions in the first peak (both A_{280} and A_{403} peaks coincide), add BSA to give a final concentration of 5 mg/mL, and store the conjugate in aliquots at −20°C (*see* **Notes 4** and **5**).

4. Notes

1. Preparations of horseradish peroxidase may vary in their carbohydrate content and this can affect the oxidation reaction. Free carbohydrate can be removed by size exclusion chromatography. Increasing the sodium periodate concentration to 0.2 *M* can also help, but further increases lead to inactivation of the peroxidase.
2. Dialysis of small volumes can be conveniently done in narrow dialysis tubing by placing a short glass tube, sealed at both ends, in the tubing so that the space available to the sample is reduced. There are also various microdialysis systems available commercially such as the Slide-A-Lyzer units from Pierce Biotechnology (Rockford, IL).
3. The absorbance at 403 nm is caused by the peroxidase's heme group. The enzyme is often specified in terms of its RZ value; this is the ratio of A_{280} and A_{403}, and it provides a measure of the heme content and purity of the preparation. Highly purified peroxidase has an RZ of about 3. Conjugates with an RZ of 0.4 perform satisfactorily.
4. BSA improves the stability of the conjugate and minimizes loses due to adsorption and denaturation. NaN_3 should not be used with peroxidase

conjugates because it inhibits the enzyme. If an antimicrobial agent is required, 0.2% sodium merthiolate (thimerosal) should be used.

5. The activity of the conjugate can be checked by the method described in **Note 4** of Chapter 64.

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Digoxigenin Labeling of IgG Antibody

G. Brian Wisdom

1. Introduction

Digoxigenin (DIG) is a plant steroid (390 Daltons) which can be used as a small, stable label of IgG molecules. It is a valuable alternative to biotin as the biotin-streptavidin system can sometimes give high backgrounds due, for example, to the presence of biotin-containing enzymes in the sample. There is a range of commercially available mouse and sheep anti-DIG Fab antibody fragments labeled with various enzymes and fluorescent molecules for the detection of the DIG-labeled IgG antibody in many applications *(**1**)*.

The IgG molecule is labeled, via its amino groups, with an *N*- hydroxysuccinimide ester derivative of the steroid containing the 6-aminocaproate spacer.

2. Materials

1. Digoxigenin-3-*O*-succinyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (Roche Applied Science, Indianapolis, IL).
2. IgG antibody. This should be the pure IgG fraction or, better, affinity purified antibody from an antiserum or pure monoclonal antibody.
3. Dimethylsufoxide (DMSO).
4. PBS: 20 m*M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* NaCl.
5. 0.1 *M* ethanolamine, pH 8.5.
6. PBS containing 0.1% bovine serum albumin (BSA).
7. Sephadex G-25 (GE Healthcare Bio-sciences, Uppsala, Sweden and Piscataway, NJ) or similar gel (*see* **Note 1**).

3. Method

1. Dissolve 1 mg of the antibody in 1 mL of PBS.
2. Prepare the digoxigenin-3-O-succinyl-*O*-minocaproic acid-*N*-hydroxysuccinimide ester immediately prior to use by dissolving it at a concentration of 2 mg/mL in DMSO.

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3. Add 24 µL of the DIG reagent to the antibody solution slowly with stirring and incubate at room temperature for 2 h.
4. Terminate the reaction by adding 0.1 mL of 0.1 *M* ethanolamine and incubate for 15 min.
5. Remove the excess DIG reagent by size-exclusion chromatography in a small column of Sephadex G-25 equilibrated with PBS containing 0.1% BSA. The IgG is in the first A_{280} peak.
6. Store the labeled antibody at 4°C with 0.05% NaN_3 or in aliquots at −20°C or lower.

4. Note

1. Suitable ready-made columns of cross-linked dextran are available from GE Biosciences (PD-10 or HiTrap columns) and from Pierce Biotechnology (Rockford, IL) (Presto and Kwik columns).

Reference

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68

Conjugation of Fluorochromes to Antibodies

Su-Yau Mao

1. Introduction

The use of specific antibodies labeled with a fluorescent dye to localize substances in tissues was first devised by A. H. Coons and his associates. At first, the specific antibody itself was labeled and applied to the tissue section to identify the antigenic sites (direct method) ***(1)***. Later, the more sensitive and versatile indirect method ***(2)*** was introduced. The primary, unlabeled, antibody is applied to the tissue section, and the excess is washed off with buffer. A second, labeled antibody from another species, raised against the IgG of the animal donating the first antibody, is then applied. The primary antigenic site is thus revealed. A major advantage of the indirect method is the enhanced sensitivity. In addition, a labeled secondary antibody can be used to locate any number of primary antibodies raised in the same animal species without the necessity of labeling each primary antibody.

Four fluorochromes are commonly used: fluorescein, rhodamine, Texas red, and phycoerythrin ***(3)***. They differ in optical properties, such as the intensity and spectral range of their absorption and fluorescence. Choice of fluorochrome depends on the particular application. For maximal sensitivity in the binding assays, fluorescein is the fluorochrome of choice because of its high quantum yield. If the ligand is to be used in conjunction with fluorescence microscopy, rhodamine coupling is advised, since it has superior sensitivity in most microscopes and less photobleaching than fluorescein. Texas red ***(4)*** is a red dye with a spectrum that minimally overlaps with that of fluorescein; therefore, these two dyes are suitable for multicolor applications. Phycoerythrin is a 240-kDa, highly soluble fluorescent protein derived from cyanobacteria and eukaryotic algae. Its conjugates are among the most sensitive fluorescent probes available ***(5)*** and are frequently used in flow cytometry and immunoassays ***(6)***. In addition, the Alexa fluorochromes are a series of fluorescent dyes with

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excitation/emission spectra similar to those of commonly used ones, but are more fluorescent and more photostable ***(7).***

Thiols and amines are the only two groups commonly found in biomolecules that can be reliably modified in aqueous solution. Although the thiol group is the easiest functional group to modify with high selectivity, amines are common targets for modifying proteins. Virtually all proteins have lysine residues, and most have a free amino terminus. The ε-amino group of lysine is moderately basic and reactive with acylating reagents. The concentration of the free-base form of aliphatic amines below pH 8.0 is very low. Thus, the kinetics of acylation reactions of amines by isothiocyanates, succinimidyl esters, and other reagents is strongly pH-dependent. Although amine acylation reactions should usually be carried out above pH 8.5, the acylation reagents degrade in the presence of water, with the rate increasing as the pH increases. Therefore, a pH of 8.5–9.5 is usually optimal for modifying lysines.

Where possible, the antibodies used for labeling should be pure. Affinity-purified, fluorochrome-labeled antibodies demonstrate less background and nonspecific fluorescence than fluorescent antiserum or immunoglobulin fractions. The labeling procedures for the isothiocyanate derivatives of fluorescein and sulfonyl chloride derivatives of rhodamine are given below ***(8).*** The major problem encountered is either over- or undercoupling, but the level of conjugation can be determined by simple absorbance readings.

2. Materials

1. IgG.
2. Borate buffered saline (BBS): 0.2 *M* boric acid, 160 m*M* NaCl, pH 8.0.
3. Fluorescein isothiocyanate (FITC) or Lissamine rhodamine B sulfonyl chloride (RBSC).
4. Sodium carbonate buffer: 1.0 *M* $NaHCO_3$-Na_2CO_3 buffer, pH 9.5, prepared by titrating 1.0 *M* $NaHCO_3$ with 1.0 *M* Na_2CO_3 until the pH reaches 9.5.
5. Absolute ethanol (200 proof) or anhydrous dimethylformamide (DMF).
6. Sephadex G-25 column.
7. Whatman DE-52 column.
8. 10 m*M* Sodium phosphate buffer, pH 8.0.
9. 0.02% Sodium azide.
10. UV spectrophotometer.

3. Methods

3.1. Coupling of Fluorochrome to IgG

1. Prior to coupling, prepare a gel-filtration column to separate the labeled antibody from the free fluorochrome after the completion of the reaction. The size of the column should be 10 bed volumes/sample volume (*see* **Note 1**).

2. Equilibrate the column in phosphate buffer. Allow the column to run until the buffer level drops just below the top of bed resin. Stop the flow of the column by using a valve at the bottom of the column.
3. Prepare an IgG solution of at least 3 mg/mL in BBS, and add 0.2 vol of sodium carbonate buffer to IgG solution to bring the pH to 9.0. If antibodies have been stored in sodium azide, the azide must be removed prior to conjugation by extensive dialysis (*see* **Note 2**).
4. Prepare a fresh solution of fluorescein isothiocyanate at 5 mg/mL in ethanol or RBSC at 10 mg/mL in DMF immediately before use (*see* **Note 3**).
5. Add FITC at a 10-fold molar excess over IgG (about 25 µg of FITC/mg IgG). Mix well and incubate at room temperature for 30 min with gentle shaking. Add RBSC at a 5-fold molar excess over IgG (about 20 µg of RBSC/mg IgG), and incubate at 4°C for 1 h.
6. Carefully layer the reaction mixture on the top of the column. Open the valve to the column, and allow the antibody solution to flow into the column until it just enters the bed resin. Carefully add phosphate buffer to the top of the column. The conjugated antibody elutes in the excluded volume (about one-third of the total bed volume).
7. Store the conjugate at 4°C in the presence of 0.02% sodium azide (final concentration) in a light-proof container. The conjugate can also be stored in aliquots at −20°C after it has been snap-frozen on dry ice. Do not refreeze the conjugate once thawed.

3.2. Calculation of Protein Concentration and Fluorochrome-to-Protein Ratio

1. Read the absorbance at 280 and 493 nm. The protein concentration is given by **Eq. 1**, where 1.4 is the optical density for 1 mg/mL of IgG (corrected to 1-cm path length).

$$\text{Fluorescein-conjugated IgG conc. (Fl IgG conc.) (mg/mL)} = (A_{280\,\text{nm}} - 0.35 \times A_{493\,\text{nm}})/1.4 \quad (1)$$

The molar ratio (F/P) can then be calculated, based on a molar extinction coefficient of 73,000 for the fluorescein group, by **Eq. 2** (*see* **Notes 4** and **5**).

$$\text{F/P} = (A_{493\,\text{nm}}/73{,}000) \times (150{,}000/\text{Fl IgG conc.}) \quad (2)$$

2. For rhodamine-labeled antibody, read the absorbance at 280 and 575 nm. The protein concentration is given by **Eq. 3**.

$$\text{Rhodamine-conjugated IgG conc. (Rho IgG conc.) (mg/mL)} = (A_{280\,\text{nm}} - 0.32 \times A_{575\,\text{nm}})/1.4 \quad (3)$$

The molar ratio (F/P) is calculated by **Eq. 4**.

$$F/P = (A_{575\ \text{nm}}/73{,}000) \times (150{,}000/\text{Rho IgG conc.}) \qquad (4)$$

4. Notes

1. Sephadex G-25 resin is the recommended gel for the majority of desalting applications. It combines good rigidity, for easy handling and good flow characteristics, with adequate resolving power for desalting molecules down to about 5000 Dalton mol wt. If the volume of the reaction mixture is <1 mL, a prepacked disposable Sephadex G-25 column (PD-10 column from Pharmacia, Piscataway, NJ) can be used conveniently.
2. When choosing a buffer for conjugation of fluorochromes, avoid those containing amines (e.g., Tris, azide, glycine, and ammonia), which can compete with the ligand.
3. Both sulfonyl chloride and isothiocyanate will hydrolyze in aqueous conditions; therefore, the solutions should be made freshly for each labeling reaction. Absolute ethanol or dimethyl formamide (best grade available, stored in the presence of molecular sieve to remove water) should be used to dissolve the reagent. The hydrolysis reaction is more pronounced in dilute protein solution and can be minimized by using a more concentrated protein solution. Caution: DMSO should not be used with sulfonyl chlorides, because it reacts with them.
4. An F/P ratio of two to five is optimal, since ratios below this yield low signals, whereas higher ratios show high background. If the F/P ratios are too low, repeat the coupling reaction using fresh fluorochrome solution. The IgG solution needs to be concentrated prior to reconjugation (e.g., Centricon-30 microconcentrator from Amicon Co., Beverly, MA, can be used to concentrate the IgG solution).
5. If the F/P ratios are too high, either repeat the labeling with appropriate changes or purify the labeled antibodies further on a Whatman DE-52 column (diethylaminoethyl microgranular preswollen cellulose, 1-mL packed column/1–2 mg of IgG). DE-52 chromatography removes denatured IgG aggregates and allows the selection of the fraction of the conjugate with optimal modification. Equilibrate and load the column with 10 m*M* phosphate buffer, pH 8.0. Wash the column with equilibrating buffer and elute with the same buffer containing 100 m*M* NaCl (first) and 250 m*M* NaCl (last). Measure the F/P ratios of each fraction, and select the appropriate fractions.
6. Alexa fluorochromes are available only as a protein labeling kit from Molecular Probes, Inc. (Eugene, Oregon, USA; www.probes.com). The reactive

dye has a succinimidyl ester moiety that reacts with primary amines of proteins. The conjugation steps are similar to those for fluorescein isothiocyanate.

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Coupling of Antibodies with Biotin

Rosaria P. Haugland and Wendy W. You

1. Introduction

The avidin–biotin bond is the strongest known biological interaction between a ligand and a protein ($K_d = 1.3 \times 10^{-15}$ *M* at pH 5) ***(1)***. The affinity is so high that the avidin–biotin complex is extremely resistant to any type of denaturing agent ***(2)***. Biotin (**Fig. 1**) is a small, hydrophobic molecule that functions as a coenzyme of carboxylases ***(3)***. It is present in all living cells. Avidin is a tetrameric glycoprotein of 66,000–68,000 mol wt, found in egg albumin and in avian tissues. The interaction between avidin and biotin occurs rapidly, and the stability of the complex has prompted its use for *in situ* attachment of labels in a broad variety of applications, including immunoassays, DNA hybridization ***(4–6)***, and localization of antigens in cells and tissues ***(7)***. Avidin has an isoelectric point of 10.5. Because of its positively charged residues and its oligosaccharide component, consisting mostly of mannose and glucosamine ***(8)***, avidin can interact nonspecifically with negative charges on cell surfaces and nucleic acids, or with membrane sugar receptors. At times, this causes background problems in histochemical and cyto-chemical applications. Streptavidin, a near-neutral, biotin binding protein ***(9)*** isolated from the culture medium of *Streptomyces avidinii,* is a tetrameric nonglycosylated analog of avidin with a mol wt of about 60,000. Like avidin, each molecule of streptavidin binds four molecules of biotin, with a similar dissociation constant. The two proteins have about 33% sequence homology, and tryptophan residues seem to be involved in their biotin binding sites ***(10, 11)***. In general, streptavidin gives less background problems than avidin. This protein, however, contains a tripeptide sequence Arg-Tyr-Asp (RYD) that apparently mimics the binding sequence of fibronectin Arg-Gly-Asp (RGD), a universal recognition domain of the extracellular matrix that specifically

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promotes cell adhesion. Consequently, the streptavidin–cell-surface interaction causes high background in certain applications ***(12)***.

As an alternative to both avidin and streptavidin, a chemically modified avidin, NeutrAvidin™ (NeutrAvidin™ is a trademark of Pierce Chemical Company, Rockford, IL) and its conjugates with enzymes or fluorescent probes are available from both Molecular Probes and Pierce. NeutrAvidin™ consists of chemically deglycosylated avidin, which has been modified to reduce the isoelectric point to a neutral value, without loss of its biotin binding properties and without significant change in the lysines available for derivatization ***(13)***. (Fluorescent derivatives and enzyme conjugates of NeutraLite avidin, as well as the unlabeled protein, are available from Molecular Probes [Eugene, OR].)

As shown in **Fig. 1**, biotin is a relatively small and hydrophobic molecule. The addition to the carboxyl group of biotin of one (*X*) or two (*XX*) aminohexanoic acid "spacers" greatly enhances the efficiency of formation of the complex between the biotinylated antibody (or other biotinylated protein) and the avidin–probe conjugate, where the probe can be a fluorochrome or an enzyme ***(14, 15)***. Each of these 7- or 14-atom spacer arms has been shown to improve the ability of biotin derivatives to interact with the binding cleft of avidin. The comparison between streptavidin binding activity of proteins biotinylated with biotin-*X* or biotin-*XX* (labeled with same number of moles of biotin/mol of protein) has been performed in our laboratory (**Fig. 2**). No difference was found between the avidin or streptavidin–horseradish peroxidase conjugates in their ability to bind biotin-*X* or biotin-*XX*. However, biotin-*XX* gave consistently higher titers in enzyme-linked immunosorbent (ELISA) assays, using biotinylated goat antimouse IgG (GAM), bovine serum albumin (BSA), or protein A (results with avidin and with protein A are not presented here). Even nonroutine conjugations performed in our laboratory have consistently yielded excellent results using biotin-*XX*.

Biotin, biotin-*X,* and biotin-*XX* have all been derivatized for conjugation to amines or thiols of proteins and aldehyde groups of glycoproteins or other

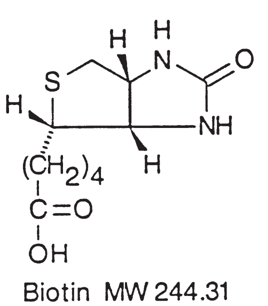

Fig. 1. Structure of biotin.

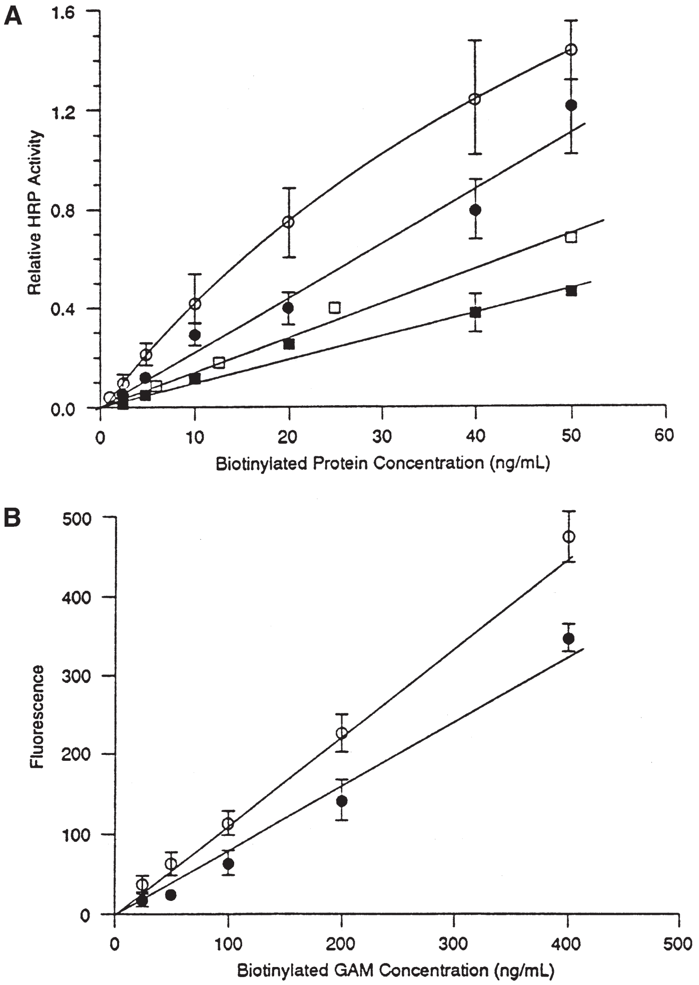

Fig. 2. (**A**) ELISA-type assay comparing the binding capacity of BSA and GAM biotinylated with biotin-*X* or biotin-*XX*. The assay was developed using streptavidin-HRP conjugate (0.2 μg/mL) and *o*-phenylenediamine dihydro-chloride (OPD). The number of biotin/mol was: 4.0 biotin-*X*/GAM (●), 4.4 biotin-*XX*/GAM (○), 6.7 biotin-*X*/BSA (■), and 6.2 biotin-*XX*/BSA (□). Error bars on some data points have been omitted for clarity. (**B**) Similar assay using GAM biotinylated with biotin-*X* (●) or biotin-*XX* (○). The assay was developed with streptavidin–R-phycoerythin conjugate (25 μg/mL using a Millipore CytoFluor™ fluorescence microtiter plate reader).

polymers. The simplest and most popular biotinylation method is to label the ε-amino groups of lysine residues with a succinimidyl ester of biotin. Easy-to-use biotinylation kits are commercially available that facilitate the biotinylation of

1–2 mg of protein or oligonucleotides ***(16)***. One kit for biotinylating smaller amounts of protein (0.1–3 mg) utilizes biotin-*XX* sulfosuccinimidyl ester ***(17)***. This compound is water-soluble and allows for the efficient labeling of dilute protein samples. Another kit uses biotin-*X* 2,4-dinitrophenyl-X-lysine succinimidyl ester (DNP-biocytin) as the biotinylating reagent. DNP-biocytin was developed by Molecular Devices (Menlo Park, CA) for their patented Threshold-Immunoligand System ***(18)***. DNP-biocytin permits the direct measurement of the degree of biotinylation of the reaction product by using the molar extinction coefficient of DNP (15,000 M^{-1} cm^{-1} at 364 nm). Conjugates of DNP-biocytin can be probed separately or simultaneously using either anti-DNP antibodies or avidin/streptavidin; this flexibility is useful when combining techniques such as fluorescence and electron microscopy. Biotin iodoacetamide or maleimide, which could biotinylate the reduced sulfhydryls located at the hinge region of antibodies, is not usually used for this purpose. More examples in the literature describe biotinylation of antibodies with biotin hydrazide at the carbohydrate prosthetic group, located in the Fc portion of the molecule, relatively removed from the binding site. Conjugation of carbohydrates with hydrazides requires the oxidation of two adjacent hydroxyls to aldehydes and optional stabilization of the reaction with cyanoborohydride ***(19)***.

Because of its strength, the interaction between avidin and biotin cannot be used for preparing matrices for affinity column purification, unless columns prepared with avidin monomers are used ***(20)***. The biotin analog, iminobiotin, which has a lower affinity for avidin, can be used for this purpose ***(21, 22)***. Iminobiotin in reactive form is commercially available, and the procedure for its conjugation is identical to that used for biotin. Detailed, practical protocols for biotinylating antibodies at the lysine or at the carbohydrate site, and a method to determine the degree of biotinylation are described in detail in this chapter (*see* **Notes 1–10** for review of factors that affect optimal conjugation and yield of biotinylated antibodies).

2. Materials

2.1. Conjugation with Amine-Reactive Biotin

1. Reaction buffer: 1 *M* sodium bicarbonate, stable for about 2 wk when refrigerated. Dissolve 8.3 g of $NaHCO_3$ in 100 mL of distilled water. The pH will be about 8.3. Dilute 1:10 before using to obtain a 0.1 *M* solution. Alternate reaction buffer: 0.1 *M* sodium phosphate, pH 7.8. Dissolve 12.7 g Na_2HPO_4 and 1.43 g NaH_2PO_4 in 800 mL of distilled water. Adjust pH to 7.8 if necessary. Bring the volume to 1000 mL. This buffer is stable for 2 mo when refrigerated.
2. Anhydrous dimethylformamide (DMF) or dimethyl sulfoxide (DMSO).
3. Phosphate-buffered saline (PBS): Dissolve 1.19 g of K_2HPO_4, 0.43 g of $KH_2PO_4{\cdot}H_2O$ and 8.8 g NaCl in 800 mL of distilled water, adjust the pH to 7.2 if necessary or to the desired pH, and bring the volume to 1000 mL with distilled water.

4. Disposable desalting columns or a gel-filtration column: Amicon GH-25 and Sephadex G-25 or the equivalent, equilibrated with PBS or buffer of choice.
5. Good-quality dialysis tubing as an alternative to the gel-filtration column when derivatizing small quantities of antibody.
6. Biotin, biotin-*X* or biotin-*XX* succinimidyl ester: As with all succinimidyl esters, these compounds should be stored well desiccated in the freezer.

2.2. Conjugation with Biotin Hydrazide at the Carbohydrate Site

1. Reaction buffer: 0.1 *M* acetate buffer, pH 6.0. Dilute 5.8 mL acetic acid in 800 mL distilled water. Bring the pH to 6.0 with 5 *M* NaOH and the volume to 1000 mL. The buffer is stable for several months when refrigerated.
2. 20 m*M* Sodium metaperiodate: Dissolve 43 mg of $NaIO_4$ in 10 mL of reaction buffer, protecting from light. Use fresh.
3. Biotin-*X* hydrazide or biotin-*XX* hydrazide.
4. DMSO.
5. Optional: 100 m*M* sodium cyanoborohydride, freshly prepared. Dissolve 6.3 mg of $NaBH_3CN$ in 10 mL of 0.1 m*M* NaOH.

2.3. Determination of the Degree of Biotinylation

1. 10 m*M* 4′ -Hydroxyazobenzene-2-carboxylic acid (HABA) in 10 m*M* NaOH.
2. 50 m*M* Sodium phosphate and 150 m*M* NaCl, pH 6.0. Dissolve 0.85 g of Na_2HPO_4 and 6.07 g of NaH_2PO_4 in 800 mL of distilled water. Add 88 g of NaCl. Bring the pH to 6.0 if necessary and the volume to 1000 mL.
3. 0.5 mg/mL Avidin in 50 m*M* sodium phosphate and 150 m*M* NaCl, pH 6.0.
4. 0.25 m*M* Biotin in 50 m*M* sodium phosphate, and 150 m*M* NaCl, pH 6.0.

3. Methods

3.1. Conjugation with Amine-Reactive Biotin

1. Calculate the amount of a 10 mg/mL biotin succinimidyl ester solution (biotin-NHS) needed to conjugate the desired quantity of antibody at the chosen biotin/antibody molar ratio, according to the following formula:

$$\text{(mL of 10 mg/mL biotin-SE)} = \{[(\text{mg antibody} \times 0.1)/\text{mol wt of antibody}] \times R \times \text{mol wt of biotin-SE})\} \tag{1}$$

where R = molar incubation ratio of biotin/protein. For example, using 5 mg of IgG and a 10:1 molar incubation ratio of biotin-*XX*-SE, **Eq. (1)** yields:

$$\text{(mL of 10 mg/mL biotin-}XX\text{-SE)} = \{[(5 \times 0.1)/145{,}000] \times (10 \times 568)\} = 0.02\ \text{mL} \tag{2}$$

2. Dissolve the antibody, if lyophilized, at approx 5–15 mg/mL in either of the two reaction buffers described in **Subheading 2.1.** If the antibody to be conjugated is already in solution in 10–20 m*M* PBS, without azide, the pH necessary for the reaction can be

obtained by adding 1/10 vol of 1 *M* sodium bicarbonate. IgM should be conjugated in PBS, pH 7.2 (*see* **Note 3**).

3. Weigh 3 mg or more of the biotin-SE of choice, and dissolve it in 0.3 mL or more of DMF or DMSO to obtain a 10 mg/mL solution. **It is essential that this solution be prepared immediately before starting the reaction,** since the succinimidyl esters or any amine-reactive reagents hydrolyze quickly in solution. Any remaining solution should be discarded.
4. While stirring, slowly add the amount of 10 mg/mL solution, calculated in **step 1**, to the antibody prepared in **step 2**, mixing thoroughly.
5. Incubate this reaction mixture at room temperature for 1 h with gentle stirring or shaking.
6. The antibody conjugate can be purified on a gel-filtration column or by dialysis. When working with a few milligrams of dilute antibody solution, care should be taken not to dilute the antibody further. In this case, dialysis is a very simple and effective method to eliminate unreacted biotin. A few mL of antibody solution can be effectively dialyzed in the cold against 1 L of buffer with three to four changes. Small amounts of concentrated antibody can be purified on a prepackaged desalting column equilibrated with the preferred buffer, following the manufacturer's directions. Five or more milligrams of antibody can be purified on a gel-filtration column. The dimensions of the column will have to be proportional to the volume and concentration of the antibody. For example, for 5–10 mg of antibody in 1 mL solution, a column with a bed vol of 10 × 300 mm will be adequate. To avoid denaturation, dilute solutions of biotinylated antibodies should be stabilized by adding BSA at a final concentration of 0.1–1%.

3.2. Conjugation with Biotin Hydrazide at the Carbohydrate Site

1. It is essential that the entire following procedure be carried out with the sample completely protected from light (*see* **Note 9**).
2. Dissolve antibody (if lyophilized) or dialyze solution of antibody to obtain a 2–10 mg/mL solution in the reaction buffer described in **Subheading 2.1., item 1**. Keep at 4°C.
3. Add an equal volume of cold metaperiodate solution. Incubate the reaction mixture at 4°C for 2 h in the dark.
4. Dialyze overnight against the same buffer protecting from light, or, if the antibody is concentrated, desalt on a column equilibrated with the same buffer. This step removes the iodate and formaldehyde produced during oxidation.
5. Dissolve 10 mg of the biotin hydrazide of choice in 0.25 mL of DMSO to obtain a 40 mg/mL solution, warming if needed. This will yield a 107 m*M* solution of biotin-*X* hydrazide or an 80 m*M* solution of biotin-*XX* hydrazide. These solutions are stable for a few weeks.
6. Calculate the amount of biotin hydrazide solution needed to obtain a final concentration of approx 5 m*M*, and add it to the oxidized antibody. When using biotin-*X* hydrazide, 1 vol of hydrazide should be added to 20 vol of antibody solution. When using biotin-*XX* hydrazide, 1 vol of hydrazide should be added to 15 vol of antibody solution.

7. Incubate for 2 h at room temperature with gentle stirring.
8. This step is optional. The biotin hydrazone–antibody conjugate formed in this reaction (**steps 6** and **7**) is considered by some researchers to be relatively unstable. To reduce the conjugate to a more stable, substituted hydrazide, treat the conjugate with sodium cyanoborohydride at a final concentration of 5 m*M* by adding a 1/20 vol of a 100-m*M* stock solution. Incubate for 2 h at 4°C (*see* **Note 5**).
9. Purify the conjugate by any of the methods described for biotinylating antibodies at the amine site (*see* **Subheading 3.1., step 6**).

3.3. Determination of the Degree of Biotinylation

The dye HABA interacts with avidin yielding a complex with an absorption maximum at 500 nm. Biotin, because of its higher affinity, displaces HABA, causing a decrease in absorbance at 500 nm proportional to the amount of biotin present in the assay.

1. To prepare a standard curve, add 0.25 mL of HABA reagent to 10 mL of avidin solution. Incubate 10 min at room temperature and record the absorbance at 500 nm of 1 mL avidin–HABA complex with 0.1 mL buffer, pH 6.0. Distribute 1 mL of the avidin–HABA complex into six test tubes. Add to each the biotin solution in a range of 0.005–0.10 mL. Bring the final volume to 1.10 mL with pH 6.0 buffer, and record the absorbance at 500 nm of each concentration point. Plot a standard curve with the nanomoles of biotin vs the decrease in absorbance at 500 nm. An example of a standard curve is illustrated in **Fig. 3.**
2. To measure the degree of biotinylation of the sample, add an aliquot of biotinylated antibody of known concentration to 1 mL of avidin–HABA complex. For example,

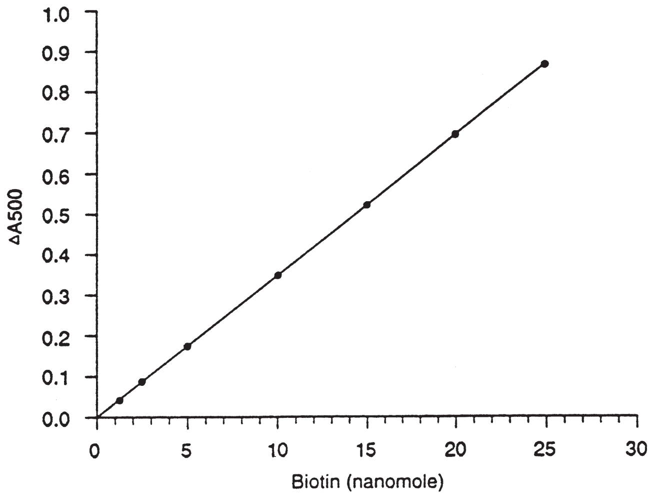

Fig. 3. Examples of standard curve for biotin assay with avidin-HABA reagent, obtained as described in **Subheading 3.3.**

add 0.05–0.1 mL of biotinylated antibody at 1 mg/mL to 1 mL of avidin–HABA mixture. Bring the volume to 1.10 mL, if necessary, incubate for 10 min, and measure the decrease in absorbance at 500 nm.

3. Deduct from the standard curve the nanomoles of biotin corresponding to the observed change in absorbance. The ratio between nanomoles of biotin and nanomoles of antibody used to displace HABA represents the degree of biotinylation, as seen from the following equation:

$$\begin{aligned}&[(\text{nmol biotin} \times 145{,}000 \times 10^{-6})/(\text{mg/mL antibody} \times 0.1\ \text{mL})] \\ &= (\text{mol of biotin/mol of antibody})\end{aligned} \tag{3}$$

where 145,000 represents the mol wt of the antibody and 0.1 mL is the volume of 1 mg/mL of biotinylated antibody sample.

4. Notes

4.1. Factors that Influence the Biotinylation Reaction

1. Protein concentration: As in any chemical reaction, the concentration of the reagents is a major factor in determining the rate and the efficiency of the coupling. Antibodies at a concentration of 5–20 mg/mL will give better results; however, it is often difficult to have such concentrations or even such quantities available for conjugation. Nevertheless, the antibody should be as concentrated as possible. In the case of solutions of antibody <2–3 mg/mL, the molar ratio of biotinylating reagent (or of both the oxidizing and biotinylating reagent, in the case of labeling the carbohydrate region) should be increased. It is also essential that the antibody solutions do not contain gelatin or BSA, which are often added to stabilize dilute solutions of antibodies. These proteins, generally present at a 1% concentration, will also react with biotinylating reagents.
2. pH: The reactivity of amines increases at basic pH. Unfortunately, so does the rate of hydrolysis of succinimidyl esters. We have found that the best pH for biotinylation of the ε-amino groups of lysines is 7.5–8.3. IgM antibodies, which denature at basic pH, can be biotinylated at pH 7.2 by increasing the molar ratio of the biotinylating reagent to antibody to at least 20. The optimum pH for oxidation and conjugation with hydrazides is 5.5–6.0.
3. Buffer: Bicarbonate or phosphate buffers are suitable for biotinylation. Organic buffers, such as Tris which contain amines, should be avoided, because they react with amino-labeling reagents or interfere with the reaction between aldehydes and hydrazides. However, HEPES and EPPS, which contain tertiary amines, are suitable. Antibodies dissolved in 10–20 m*M* PBS can be readily prepared for conjugation at the lysine site by adding 1/10–1/5 of the volume of 1 *M* sodium bicarbonate. As noted, because IgM antibodies are unstable in basic solution, biotinylation at the ε-amino group

of lysines should be attempted in PBS or equivalent buffer at pH 7.2. Reactions of antibodies with periodate and biotin hydrazide can be performed in PBS at pH 7 or in acetate buffer, pH 6.0 (*see* **Subheading 2.2**).

4. Temperature: Biotinylations at the amino group sites are run at room temperature, at the carbohydrate site at 0–4°C.
5. Time: Succinimidyl ester derivatives will react with a protein within 1 h. Periodate oxidation will require 2 h at pH 6.0. Reaction with biotin hydrazide can be performed in a few hours. Stabilization with cyanoborohydride requires <2 h.
6. Desired degree of biotinylation and stability of the conjugate: Reaction of an antibody with biotin does not significantly alter the size or charge of the molecule. However, because of the size of avidin or its analogs (mol wt = 60,000–68,000), an increase in the number of biotins per antibody will not necessarily increase the number of avidins capable of reacting with one antibody molecule. Because biotin, biotin-*X*, and biotin-*XX* are very hydrophobic molecules, a high degree of biotinylation might increase the background or might destabilize the antibody. To obtain a degree of biotinylation of about 3–7 biotins/IgG, generally a molar ratio of 15 mol of amino biotinylating reagent/mol of protein is used. When the concentration of the antibody is <3 mg/mL, this ratio should be increased. The amount of increase should be determined experimentally, because the reactivity of the lysines available for conjugation varies for each antibody (Ab). This could become a significant factor, especially at low antibody concentrations.

The succinimidyl esters or hydrazides of biotin, biotin-*X*, and biotin-*XX* exhibit similar degrees of reactivity, and the choice is up to the researcher. In general, the longer spacer arm in biotin-*XX* should be advantageous (**Fig. 2**). The overall stability of biotinylated MAbs derivatized with a moderate number of biotin should be similar to the stability of the native antibody, and the storage conditions also should be the same.

4.2. Factors that Affect Antibodies

7. Most Abs can withstand biotinylation with minimal change in activity and stability, especially if the degree of biotinylation is about 3–6 biotins/mol.
8. Biotin or any of its longer chain derivatives do not contribute to the absorbance of the antibody at 280 nm. Consequently, the concentration of the antibody can be measured by using $A^{1\%}_{1\,\text{cm}} = 14$ at 280 nm.
9. It is essential that the entire procedure for biotinylation of antibodies at the carbohydrate site (**Subheading 3.2**) be performed in the dark, protected from light.
10. It should be noted that dry milk, serum, and other biological fluids contain biotin and, consequently, they should not be used as blocking agents in systems where blocking is required.

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Preparation of Avidin Conjugates

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1. Introduction

The high-affinity avidin–biotin system has found applications in different fields of biotechnology including immunoassays, histochemistry, affinity chromatography, and drug delivery, to name a few. A brief description of avidin and avidin-like molecules, streptavidin, deglycosylated avidin, and NeutrAvidin™ avidin, is presented in Chapter 69. With four biotin binding sites per molecule, the avidin family of proteins is capable of forming tight complexes with one or more biotinylated compounds ***(1)***. Typically, the avidin–biotin system is used to prepare signal-amplifying "sandwich" complexes between specificity reagents (e.g., antibodies) and detection reagents (e.g., fluorophores, enzymes, and so on). The specificity and detection reagents are independently conjugated, one with avidin and the other with biotin, or both with biotin, providing synthetic flexibility ***(2)***.

Avidin conjugates of a wide range of fluorophores, phycobiliproteins, secondary antibodies, microspheres, ferritin, and enzymes commonly used in immunochemistry are available at reasonable prices, making their small scale preparation impractical and not cost effective (*see* **Note 1**). However, conjugations of avidin to specific antibodies, to uncommon enzymes, and to other proteins and peptides are often performed onsite. A general protocol for the conjugation of avidin to enzymes, antibodies, and other proteins is described in this chapter.

Avidin conjugates of oligodeoxynucleotides are hybrid molecules that not only provide multiple biotin binding sites, but can also be targeted to complementary DNA or RNA sequences, by annealing interactions. Such conjugates are useful for the construction of macromolecular assemblies with a wide variety of

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constituents *(3)*. The protocol outlined in **Subheading 3.1** can be modified (*see* **Note 2**) for the conjugation of oligonucleotides to avidin.

Streptavidin conjugates are also being evaluated for use in drug delivery systems. A two-step imaging and treatment protocol has been developed that involves injection of a suitably prepared tumor-specific monoclonal antibody, followed by a second reagent that carries an imaging or therapeutic agent, capable of binding to the tumor-targeted antibody *(4)*. Owing to complications associated with the injection of radiolabeled biotin *(5)*, conjugation of the imaging or therapeutic agent to streptavidin is being considered, instead. A protocol for radioiodination of streptavidin using IODO-BEADS® *(6)* is described in **Subheading 3.2**. Some other methods that have been developed include the iodogen method *(7,8)*, (*see* Chapter 176), the Bolton–Hunter reagent method (*see* Chapter 177) *(9)*, and a few that do not involve direct iodination of tyrosine residues *(10–13)*. Streptavidin–drug conjugates are also candidates for therapeutic agents. Synthesis of a Streptavidin-drug conjugate involves making a chemically reactive form of the drug followed by its conjugation to streptavidin. The synthetic methodology thus depends on the structure of the specific drug to be conjugated *(14–16)*.

The avidin-biotin interaction can also be exploited for affinity chromatography; however, there are limitations to this application, because a biotinylated protein captured on an avidin affinity matrix would likely be denatured by the severe conditions required to separate the high-affinity avidin–biotin complex. On the other hand, an avidin affinity matrix may find utility in the removal of undesired biotinylated moieties from a mixture or for the purification of compounds derivatized with 2-iminobiotin. The biotin derivative 2-iminobiotin has reduced affinity for avidin, and its moderate binding to avidin at pH 9.0 is greatly diminished at pH 4.5 *(17)*. Another approach to reducing the affinity of the interaction is to denature avidin to its monomeric subunits. The monomeric subunits have greatly reduced affinity for biotin *(18)*. We describe here a protocol for preparing native *(19)* and monomeric avidin matrices *(20)*. Modified streptavidins, hybrids of native and engineered subunits with lower binding constants, have been prepared that may also be suitable for affinity matrices *(21)*. A new form of monovalent avidin, nitrated at the tyrosine located at three of the four biotin binding sites, has recently been available from Molecular Probes. The binding affinity of this modified avidin *(22)*, called CaptAvidin™ biotin-binding protein, is lower than for the native protein. At pH 4 CaptAvidin biotin-binding protein associates with biotin with a K_a of 10^9 M^{-1}; if needed, the complex can be completely dissociated at pH ~10.0. This property makes CaptAvidin biotin-binding protein the ideal ligand for affinity matrices suitable for isolation and purification of biotinylated compounds. A nitration protocol is described in Chapter 87.

2. Materials

2.1. Conjugation to Antibodies, Enzymes or Oligonucleotides

1. Avidin (mol wt = 66,000).
2. Antibody, enzyme, peptide, protein, or thiolated oligonucleotide to be conjugated to avidin.
3. Succinimidyl 3-(2-pyridyldithio)propionate (SPDP; mol wt = 312.36) (*see* **Note 3**).
4. Succinimidyl *trans*-4-(*N*-maleimidylmethyl)cyclohexane-1-carboxylate (SMCC; mol wt = 334.33).
5. Dithiothreitol (DTT; mol wt = 154.24).
6. Tris-(2-carboxyethyl) phosphine (TCEP; mol wt = 286.7).
7. *N*-ethylmaleimide (NEM; mol wt = 125.13)
8. Anhydrous dimethyl sulfoxide (DMSO) or anhydrous dimethylformamide (DMF).
9. 0.1 *M* Phosphate buffer: Contains 0.1 *M* sodium phosphate, 0.1 *M* NaCl at pH 7.5. Dissolve 92 g of Na_2HPO_4, 21 g of $NaH_2PO_4 \bullet H_2O$, and 46.7 g of NaCl in approx 3.5 L of distilled water and adjust the pH to 7.5 with 5 *M* NaOH. Dilute to 8 L. Store refrigerated.
10. 1 *M* Sodium bicarbonate (*see* **Note 4**). Dissolve 8.4 g in 90 mL of distilled water and adjust the volume to 100 mL. A freshly prepared solution has a pH of 8.3–8.5.
11. Molecular exclusion matrix with properties suitable for purification of the specific conjugate. Sephadex G-200 (Pharmacia Biotech, Uppsala, Sweden), Bio-Gel® A-0.5 m or Bio-Gel® A-1.5 m (Bio-Rad Laboratories, Hercules, CA) are useful for relatively small to large conjugates, respectively.
12. Sephadex G-25 (Pharmacia Biotech) or other equivalent matrix

2.2. Radioiodination Using IODO-BEADS

1. Streptavidin (mol wt = 60,000).
2. $Na^{131}I$ or $Na^{125}I$, as desired.
3. IODO-BEADS (Pierce Chemical, Rockford, IL).
4. Phosphate-buffered saline (PBS), pH 7.2 : Dissolve 1.19 g of K_2HPO_4, 0.43 g of KH_2PO_4, and 9 g of NaCl in 900 mL of distilled water. Adjust the pH to 7.2 and dilute to 1 L with distilled water.
5. Saline solution: 9 g of NaCl dissolved in 1 L of distilled water.
6. 0.1% Bovine serum albumin (BSA) solution in saline: 0.1 g of bovine serum albumin dissolved in 100 mL of saline solution.
7. Trichloroacetic acid (TCA), 10% w/v solution in saline: Dissolve 1 g of TCA in 10 mL of saline solution.
8. Bio-Gel® P-6DG Gel (Bio-Rad Laboratories).

2.3. Avidin Affinity Matrix

1. 50–100 mg of avidin.
2. Sodium borohydride.
3. 1,4-Butanediol-diglycidyl ether.
4. Succinic anhydride.

5. 6 *M* Guanidine HCl in 0.2 *M* KCl/HCl, pH 1.5: Dissolve 1.5 g of KCl in 50 mL of distilled water. Add 57.3 g of guanidine HCl with stirring. Adjust the pH to 1.5 with 1 *M* HCl. Adjust the volume to 100 mL with distilled water.
6. 0.2 *M* Glycine HCl pH 2.0: Dissolve 22.3 g of glycine HCl in 900 mL of distilled water. Adjust the pH to 2.0 with 6 *M* HCl and the volume to 1 L with distilled water.
7. PBS: *See* **Subheading 2.2.4.**
8. 0.2 *M* Sodium carbonate, pH 9.5: Dissolve 1.7 g of sodium bicarbonate in 80 mL of distilled water. Adjust the pH to 9.5 with 1 *M* NaOH and the volume to 100 mL with distilled water.
9. 0.2 *M* Sodium phosphate, pH 7.5: Weigh 12 g of Na_2HPO_4 and 2.5 g of $NaH_2PO_4 \cdot H_2O$ and dissolve in 900 mL of distilled water. Adjust the pH to 7.5 with 5 *M* NaOH and the volume to 1 L with distilled water.
10. 20 m*M* Sodium phosphate, 0.5 *M* NaCl, 0.02% sodium azide pH 7.5: Dilute 100 mL of the buffer described in **Subheading 2.3., item 9**) to 900 mL with distilled water. Add 28 g of NaCl and 200 mg of sodium azide. Adjust pH if necessary, and dilute to 1 L with distilled water.
11. Sepharose 6B (Pharmacia Biotech) or other 6% crosslinked agarose gel.

3. Methods

3.1. Conjugation to Antibodies or to Enzymes

3.1.1. Avidin Thiolation

An easy-to-use, protein-to-protein crosslinking kit is now commercially available (Molecular Probes, Eugene, OR). This kit allows predominantly 1:1 conjugate formation between two proteins (0.2–3 mg) through the formation of a stable thioether bond ***(23)***, with minimal generation of aggregates. A similar protocol is described here for conjugation of 5 mg of avidin to antibodies or enzymes. Modifications of the procedure for conjugation of avidin to thiolated oligonucleotides and peptides are described in **Notes 2** and **5**, respectively. Although the protocol described in this section uses avidin for conjugation, it can be applied for the preparation of conjugates using either avidin, streptavidin, deglycosylated avidin, or NeutrAvidin avidin.

1. Dissolve 5 mg of avidin (76 nanomol) in 0.5 mL of 0.1 *M* phosphate buffer to obtain a concentration of 10 mg/mL.
2. Weigh 3 mg of SPDP and dissolve it in 0.3 mL of DMSO to obtain a 10 mg/mL solution. This solution must be prepared **fresh** immediately before using. Vortex-mix or sonicate to ensure that the reagent is completely dissolved.
3. Slowly add 12 µL (380 nanomoles) of the SPDP solution (*see* **Note 3**) to the stirred solution of avidin. Stir for 1 h at room temperature.
4. Purify the thiolated avidin on a 7 × 250 mm size exclusion column, such as Sephadex G-25 equilibrated in 0.1 *M* phosphate buffer.
5. Determine the degree of thiolation (optional):

Table 1
Molar Extinction Coefficients at 280 nm and Molecular Weights of Avidin and Avidin-Like Proteins

Protein	Molecular weight	E_{Avidin}^{M}/cm/M
Avidin	66,000	101,640
Deglycosylated avidin/NeutrAvidin	60,000	101,640
CaptAvidin biotin-binding protein	66,000	118,800
Streptavidin	60,000	180,000

a. Prepare a 100 m*M* solution of DTT by dissolving 7.7 mg of the reagent in 0.5 mL of distilled water.

b. Transfer the equivalent of 0.3–0.4 mg of thiolated avidin (absorbance at 280 nm of a 1.0 mg/mL avidin solution = 1.54) and dilute to 1.0 mL using 0.1 *M* phosphate buffer. Record the absorbance at 280 nm and at 343 nm.

c. Add 50 mL of DTT solution. Mix well, incubate for 3–5 min at room temperature and record the absorbance at 343 nm.

d. Using the extinction coefficient at 343 nm of 8.08×10^3/cm/M ***(24)***, calculate the amount of pyridine-2-thione liberated during the reduction, which is equivalent to the number of thiols introduced on avidin, using the following equation along with the appropriate extinction coefficient shown in **Table 1**:

$$\text{Number of thiols/avidin} = [\triangle A_{343}/(8.08 \times 10^3)] \times (A_{280} - 0.63\triangle A_{343})] \qquad (1)$$

where ΔA_{343} = change in absorbance at 343 nm; E^{M}_{avidin} = molar extinction coefficient; and $0.63\Delta A_{343}$ = correction for the absorbance of pyridyldithiopropionate at 280 nm ***(24)***.

6. **Equation 1** allows the determination of the average number of moles of enzyme or antibody that can be conjugated with each mole of avidin (*see* **Note 6**). For a 1:1 proteinavidin conjugate, avidin should be modified with 1.2–1.5 thiols/mol. Thiolated avidin prepared by the above procedure can be stored in the presence of 2 m*M* sodium azide at 4°C for 4–6 wk.

3.1.2. Maleimide Derivatization of the Antibody or Enzyme

In this step, which should be completed prior to the deprotection of thiolated avidin, some of the amino groups from the antibody or enzyme are transformed into maleimide groups by reacting with the bifunctional crosslinker, SMCC. (*see* **Note 7**).

1. Dissolve or, if already in solution, dialyze the protein in 0.1 *M* phosphate buffer to obtain a concentration of 2–10 mg/mL. If the protein is an antibody, 11 mg are required to obtain an amount equimolar to 5 mg of avidin (*see* **Note 6**).

2. Prepare a **fresh** solution of SMCC by dissolving 5 mg in 0.5 mL of dry DMSO to obtain a 10 mg/mL solution. Vorte-mix or sonicate to assure that the reagent is completely dissolved.
3. While stirring, add an appropriate amount of SMCC solution to the protein solution to obtain a molar ratio of SMCC to protein of approx 10. (If 11 mg of an antibody is the protein used, 30 µL of SMCC solution is required.)
4. Continue stirring at room temperature for 1 h.
5. Dialyze the solution in 2 L of 0.1 *M* phosphate buffer at 4°C for 24 h, with four buffer changes using a membrane with a suitable molecular weight cutoff.

3.1.3. Deprotection of the Avidin Thiol Groups

This procedure is carried out immediately before reacting thiolated avidin with the maleimide derivative of the antibody or enzyme prepared in **Subheading 3.1.2.**

1. Dissolve 3 mg of TCEP in 0.3 mL of 0.1 *M* phosphate buffer.
2. Add 11 µL of TCEP solution to the thiolated avidin solution. Incubate for 15 min at room temperature.

3.1.4. Formation and Purification of the Conjugate

1. Add the thiolated avidin–TCEP mixture dropwise to the dialyzed maleimide derivatized protein solution with stirring. Continue stirring for 1 h at room temperature, followed by stirring overnight at 4°C.
2. Stop the conjugation reaction by capping residual sulfhydryls with the addition of NEM at a final concentration of 50 µ*M*. Dissolve 6 mg of NEM in 1 mL of DMSO and dilute 1:1000 in the conjugate reaction mixture. Incubate for 30 min at room temperature or overnight at 4°C (*see* **Note 8**). The conjugate is now ready for final purification.
3. Concentrate the avidin–protein conjugate mixture to 1–2 mL in a Centricon®-30 (Amicon, Beverly, MA) or equivalent centrifuge tube concentrator.
4. Pack appropriate size columns (e.g., 10 × 60 mm for approx 15 mg of final conjugate) with a degassed matrix suitable for the isolation of the conjugate from unconjugated reagents. If the protein conjugated is an antibody, a matrix such as Bio-Gel A-0.5 m is suitable. For other proteins, Sephadex G-200 or a similar column support may be appropriate, depending on the size of the protein–avidin conjugate.
5. Collect 0.5–1-mL fractions. The first protein peak to elute contains the conjugate, however the first or second fraction may contain some aggregates. Analyze each fraction absorbing at 280 nm for biotin binding and assay it for the antibody or enzyme activity. High-performance liquid chromatography (HPLC) may be also be performed for further purification, if necessary.

3.2. Radioiodination Using IODO-BEADS

The radioiodination procedure (*see* **Note 9**) described here uses IODO-BEADS, which contain the sodium salt of *N*-chlorobenzenesulfonamide immobilized on nonporous, polystyrene beads. Immobilization of the oxidizing agent allows for easy separation of the latter from the reaction mixture. This method also avoids the use of reducing agents.

1. Wash six to eight IODO-BEADS twice with 5 mL of PBS. Dry the beads by rolling them on a clean filter paper.
2. Add 500 μL of PBS to the supplier's vial containing 8–10 mCi of carrier-free $Na^{125}I$ or $Na^{131}I$. Place the beads in the same vial and gently mix the contents by swirling. Allow the mixture to sit for 5 min at room temperature with the vial capped.
3. Dissolve or dilute streptavidin in PBS to obtain a final concentration of 1 mg/mL. Add 500 μL of streptavidin solution to the vial containing sodium iodide. Cap the vial immediately and mix the contents thoroughly. Incubate for 20–25 min at room temperature, with occasional swirling (*see* **Note 10**).
4. Carefully remove and save the liquid from the reaction vessel; this is the radioiodinated streptavidin solution. Wash the beads by adding 500 μL of PBS to the reaction vial. Remove the wash solution and add it to the radioiodinated streptavidin.
5. For purification, load the reaction mixture onto a 9 × 200 mm Bio-Gel P-6DG column packed in PBS (0.1% BSA may be added as a carrier to the PBS to reduce loss of streptavidin by adsorption to the column). Elute the column with PBS and collect 0.5-mL fractions. The first set of radioactive fractions (as determined by counting in a γ-ray counter) contain radioiodinated streptavidin, while the unreacted radioiodine elutes in the later fractions. Pool the radioiodinated streptavidin fractions.
6. Assessment of protein-associated activity with TCA acid precipitation:
   a. Dilute a small volume of the pooled radiolabeled streptavidin with saline solution such that 50 μL of the diluted solution has 10^4–10^6 cpm.
   b. Add 50 μL of the diluted streptavidin solution to a 12 mm × 75 mm glass tube, followed by 500 μL of a 0.1% BSA solution in saline.
   c. For precipitating the proteins, add 500 μL of 10% (w/v) TCA solution in saline.
   d. Incubate the solution for 30 min at room temperature and count the radioactivity of the solution for 10 min ("Total Counts").
   e. Centrifuge the tube at 500 g for 10 min and carefully discard the supernatant in a radioactive waste container.
   f. Resuspend the pellet in 1 mL of saline and count its radioactivity for 10 min ("Bound Counts").
   g. The percentage of radioactivity bound to streptavidin is determined using the following equation:

$$[(\text{Bound counts})/(\text{Total counts})] \times 100 = \%\ \text{of radioactivity bound to streptavidin} \quad (2)$$

3.3. Avidin Affinity Matrices

3.3.1. CaptAvidin Biotin-Binding Protein Affinity Matrix

CaptAvidin agarose is available from Molecular Probes. For the preparation of this column avidin has been nitrated at the tyrosine sites involved with biotin binding. Nitration is performed to the extent that three of the four active sites of avidin are modified and loose their binding activity for biotin. The fourth site allows binding of biotin at pH 4 with a K_a of 10^9 M^{-1} as reported by Morag et al. ***(22)***. This monovalent form of modified avidin had been covalently attached to agarose to generate an affinity matrix that does not need the harsh eluting conditions necessary for the avidin or even the iminobiotin affinity columns. The biotinylated compounds are easily dissociated from the CaptAvidin, biotin-binding protein matrix by elution at pH 10.0.

3.3.2. Native Avidin Affinity Matrix

1. Wash 10 mL of sedimented 6% crosslinked agarose with distilled water on a glass or Buchner filter and remove excess water by suction.
2. Dissolve 14 mg of $NaBH_4$ in 7 mL of 1 *M* NaOH. Add this solution along with 7 mL of 1,4-butanediol-diglycidyl ether to the washed agarose, with mixing. Allow the reaction to proceed for 10 h or more at room temperature with gentle stirring.
3. Extensively wash the activated gel with distilled water on a supporting filter. The washed gel can be stored in water at 4°C, for up to 10 d.
4. Dissolve 50–100 mg of avidin in 10–20 mL of 0.2 *M* sodium carbonate, pH 9.5, and suspend the sedimented activated agarose gel in the same buffer to obtain a workable slurry.
5. Slowly drip the agarose slurry into the stirred protein solution and allow the binding to take place at room temperature for 2 d with continuous gentle mixing.
6. Wash the avidin–agarose mixture in PBS until the filtrate shows no absorbance at 280 nm. Store at 4°C in the presence of 0.02% sodium azide.

3.3.3. Monomeric Avidin Affinity Matrix

1. Filter the avidin–agarose matrix (from **Subheading 3.3.1., step 6**) on a glass or Buchner filter (or pack in a column) and wash 4× with two volumes of 6 *M* guanidine HCl in 0.2 *M* KCl, pH 1.5, to dissociate the tetrameric avidin.
2. Thoroughly wash the gel with 0.2 *M* potassium phosphate, pH 7.5, and suspend in 10 mL of the same buffer.
3. Add 3 mg of solid succinic anhydride to succinylate the monomeric avidin and incubate for 1 h at room temperature with gentle stirring.
4. Wash the gel with 0.2 *M* potassium phosphate, pH 7.5, pack in a column, and saturate the binding sites by running through three volumes of 1 m*M* biotin dissolved in the same buffer.
5. Remove biotin from the low-affinity binding sites by washing the column with 0.2 *M* glycine HCl, pH 2.0.
6. Store the column equilibrated in 20 m*M* sodium phosphate, 0.5 *M* NaCl, 0.02% sodium azide, pH 7.5. The column is now ready to use.
7. Load the column with the mixture to be purified. Elute any unbound protein by adding 20 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7.5. Add biotin to the same buffer to obtain a final concentration of 0.8 m*M* to elute the biotinylated compound.
8. Regenerate the column after each run by washing with 0.2 *M* glycine HCl, pH 2.0.

4. Notes

1. A detailed procedure for the conjugation of fluorophores to antibodies has been recently published ***(25)***. This protocol can be modified for conjugation of fluorophores to avidin or avidin-related proteins by using a dye to avidin molar ratio of 5–8:1.
2. The conjugation reaction for oligonucleotides synthesized with a disulfide containing a protecting group, should be performed under nitrogen or argon. Deprotect the disulfide of the oligonucleotide using DTT. Add 1 mg of DTT to 140 µL of a 6 µ*M* oligonucleotide (21–33 mer) solution in 0.1 *M* phosphate buffer containing 5 m*M* ethylenediami–netetraacetic acid. Stir the solution at 37°C for 0.5 h. Purify the reaction mixture using a disposable desalting column. Combine the oligonucleotide-containing fractions with thiolated avidin prepared as described in **Subheading 3.1.1**. It should be noted that, in this case, conjugation occurs through the formation of a disulfide bond instead of a thioether bond. Disulfides are sensitive to reducing agents; however, they make reasonably stable conjugates, useful in most applications ***(26)***. Purify the conjugate as outlined in **Subheading 3.1.4**.
3. Using a molar ratio of SPDP to avidin of 5 yields one or two protected sulfhydryls per molecule of avidin. This range of thiols per mole is found to produce the best yield of a 1:1 conjugate.
4. Buffer and pH: The entire procedure for preparation of conjugates through thioether bonds can be performed at pH 7.5. (**Note:** Organic buffers containing amines, such as Tris, are unsuitable.) Antibodies or enzymes in PBS can be prepared for reaction with SMCC by adding 1/10 volume of 1 *M* sodium bicarbonate solution. This step eliminates dialysis and consequent dilution of the protein. The presence of azide at concentrations above 0.1% may interfere with the reaction of the protein with SMCC or of avidin with SPDP. Some gM antibodies denature above pH 7.2. They can, however, be conjugated in PBS at pH 7.0 by increasing the molar ratio of maleimide to antibody.
5. Peptides (20–25 amino acids) containing a single cysteine can also be conjugated to thiolated avidin by modifying the procedure described in **Subheading 3.1** and performing the reaction under argon or nitrogen ***(26)***. Peptide–avidin conjugate formation described here also involves the formation of a disulfide bond. For conjugation with 5 mg of avidin, dissolve 1.6 mg of a lyophilized cysteine-containing peptide in 900 µL of water–methanol (2:1 v/v) using 50 m*M* NaOH (a few microliters at a time) to improve solubility. Immediately prior to use, cleave any cystine-bridged homodimer that may be present by the addition of TCEP solution (10 mg/mL in 0.1 *M* phosphate buffer) to obtain a TCEP to peptide ratio of 3. Incubate for 15 min at room temperature. Purify the peptide–TCEP mixture using a disposable desalting column. Combine the peptide-containing

fractions with thiolated avidin prepared as described in **Subheading 3.1.1**. Purify the conjugate as described in **Subheading 3.1.4**.

6. Avidin and antibody or enzyme concentration: The concentration of avidin as well as that of the protein to be conjugated should be 2–10 mg/mL. The crosslinking efficiency and, consequently, the yield of the conjugate decreases at lower concentrations of the thiolated avidin and maleimide-derivatized protein. To obtain 1:1 conjugates, equimolar concentrations of avidin and the protein are desirable. However, most methods of conjug'ation will generate conjugates of different sizes, following the Poisson distribution. The size range obtained with the method described here is much narrower because the number of proteins reacting with each mole of avidin can be regulated by the degree of thiolation of avidin.
7. It is essential that the procedure described in **Subheading 3.1.2** be performed approx 24 h before the procedure described in **Subheading 3.1.3**, because the deprotected thiolated avidin and the maleimide derivative of the protein are unstable. Purification of the maleimide-derivatized protein by size exclusion chromatography can be performed more rapidly than dialysis; however, the former leads to dilution of the protein and a decrease in the yield of the conjugate.
8. If the molecule being conjugated to avidin is β-galactosidase or other free thiol-containing oligonucleotide or protein, NEM treatment is not performed.
9. Radioiodination of streptavidin uses procedures similar to those used for stable nuclides. However, some distinct differences remain, since radioiodinations are performed in dilute solutions. Also, the radioiodination mixture contains minor impurities formed during the preparation and purification of the radionuclide. Thus, optimization of reaction parameters is essential for performing radioiodination. This reaction is carried out in small volumes; it is therefore essential to ensure adequate mixing at the outset of the reaction. Inadequate mixing is often responsible for poor radioiodination yield.
10. Specific activity using the method described in **Subheading 3.2** is usually in the range of 10–50 mCi/mg and the protein-bound radioactivity obtained is >95%. Higher specific activity can be achieved by increasing the reaction time of **step 3** in **Subheading 3.2** by using more beads or by increasing the amount of radioiodine. However, one must bear in mind that at longer incubation times, the risk of damage to streptavidin or avidin is greater.
11. Storage and stability of avidin conjugates: Most avidin conjugates can be stored at 4°C or −20°C after lyophilization. Because of the variation in antibody structure, there is no general rule on the best method to store avidin–antibody conjugates, and the best conditions are determined experimentally.

Aliquoting in small amounts and freezing is generally satisfactory. Radiolabeled streptavidin is aliquoted (100 µL/tube) and stored at 4°C or −20°C until use.

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MDPF Staining of Proteins on Western Blots

F. Javier Alba and Joan-Ramon Daban

1. Introduction

We describe a method for the detection of total protein patterns on polyvinylidene difluoride (PVDF) membranes using the fluorogenic dye 2-methoxy-2,4-diphenyl-3(2*H*)-furanone (MDPF) ***(1)***. This method is based on the fluorescent properties of this dye ***(2)***. As can be seen in **Fig. 1**, MDPF (**A**) and the hydrolysis product (**C**) are nonfluorescent; only the adduct **B** formed with the proteins is fluorescent. This makes unnecessary the destaining of the PVDF membrane after protein labeling. The whole process of staining with MDPF is completed in about 20 min. Wet membranes are translucent, allowing the visualization of MDPF labeled protein bands by transillumination with UV light (*see* **Fig. 2A**). Electrophoretic bands containing less than 10 ng of protein transferred to PVDF membranes can be detected after the reaction with MDPF.

This staining method is compatible with previous visualization of protein bands on the sodium dodecyl sulfate (SDS)-polyacrylamide gel with the noncovalent fluorescent dye Nile red (*see* Chapter 45). Thus, Nile red and MDPF staining can be performed sequentially. This allows the rapid monitoring of total protein patterns on both the electrophoretic gel and Western blot. In addition, MDPF staining allows further immunodetection of specific bands with polyclonal antibodies (*see* **Fig. 2B**). Finally, using the adequate conditions described in the Materials section, MDPF staining does not preclude the N-terminal sequence analysis of proteins in selected bands. A review about the psychochemical basis of MDPF staining has been published elsewhere ***(3)***.

2. Materials

All solutions should be prepared using electrophoresis-grade reagents and deionized water and stored at room temperature. Wear gloves to handle all

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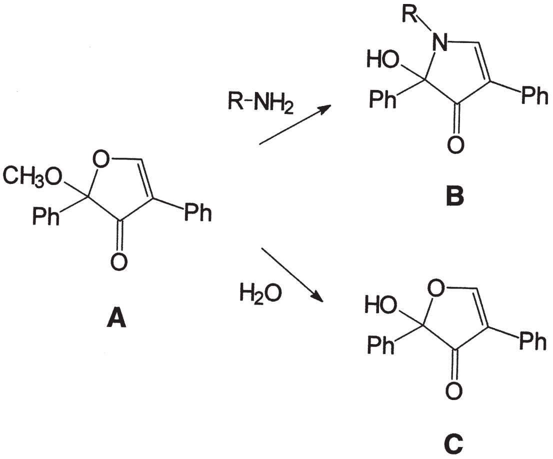

Fig. 1. According to Weigele et al. *(2)*, the reaction of MDPF (**A**) with primary amino groups of proteins produces the fluorescent adduct (**B**); the excess reagent is hydrolyzed forming the nonfluorescent product (**C**).

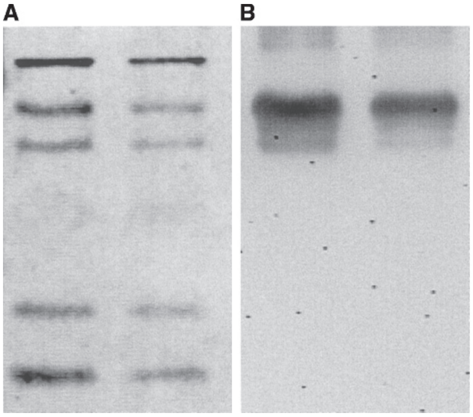

Fig. 2. (**A**) Example of MDPF staining of different proteins on PVDF membranes. The blot was equilibrated twice (5 min each time) in borate buffer and stained for 10 min with MDPF (*see* **Subheading 3.**). Proteins, from top to bottom, are: BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, β-lactoglobulin, and α-lactalbumin; the amount of each protein loaded initially onto the SDS gel before electroblotting was 100 *(left lane)* and 50 *(right lane)* ng. (**B**) After staining with MDPF ovalbumin was immunodetected with the ECL system (Amersham).

reagents and solutions and do not pipet by mouth. Collect and dispose all waste according to good laboratory practice and waste disposal regulations.

1. MDPF: Concentrated stock (35 m*M*) in dimethyl sulfoxide (DMSO). This solution is stable for 1 wk at room temperature in a glass bottle wrapped in aluminum foil. **Handle this solution with care, DMSO is flammable and, in addition, this solvent may facilitate the passage of hazardous chemicals such MDPF through the skin.** MDPF can be obtained from Fluka (Bunch, Switzerland).
2. PVDF membranes (Bio-Rad Laboratories [Hercules, CA]).
3. Transfer apparatus (e.g., Mini-Trans-Blot Cell [Bio-Rad]).
4. Opaque plastic box for membrane equilibration and staining.
5. Orbital shaker.
6. Transilluminator equipped with midrange ultraviolet (UV) bulbs (~300 nm) to excite MDPF labeled proteins on blots (e.g., Foto UV 300 [Fotodyne Inc., Harland, WI] or the transilluminator included in the Gel Doc 1000 system from Bio-Rad). *See* Chapter 45 for details about protection from UV during transillumination.
7. Photography: We have used the Polaroid (Cambridge, MA) MP-4 camera with Polaroid instant film 667 and the CCD camera of the Gel Doc 1000 system (Bio-Rad). With both cameras use the Wratten (Kodak [Rochester, NY]) filters numbers 3 (yellow) and 47 (blue). Place filter 47 on top of filter 3. Store the filters in the dark and protect them from heat, intense light sources, and humidity. Store the Polaroid film at 4°C.
8. Immunodetection: Ovalbumin antiserum developed in rabbit (Sigma [St. Louis, MO]); ECL detection kit, including horseradish peroxirase-labeled antirabbit antibodies (Amersham [Buckinghamshire, UK]).
9. Transfer buffer: 25 m*M* Tris, 192 m*M* glycine, pH 8.3.
10. Borate buffer: 10 m*M* sodium borate, pH 9.5.
11. Phosphate-buffered saline: 80 m*M* Na_2HPO_4, 20 m*M* NaH_2PO_4, 100 m*M* NaCl, pH 7.4.

3. Method

After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the gels were stained with Nile red (*see* Chapter 45). Protein bands stained with Nile red can be transferred to PVDF membranes without being necessary to perform the destaining of the gel ***(1)***. The method described in this part gives all the details for the staining of the blotted proteins with MDPF. All operations are performed at room temperature. Handle blots by their edges using stainless steel forceps.

1. After electrophoresis and Nile red staining, equilibrate the gel (8 × 6 × 0.075 cm) in 100 mL of transfer buffer for 15 min. Immerse sequentially the PVDF membrane in 20 mL of methanol for 5 s, in 100 mL of water for 2–3 min, and finally in 100 mL of transfer buffer for 10 min.
2. Assemble the gel and the membrane in the blotting apparatus and fill the tank with transfer buffer. Perform electroblotting at 100 V for 1 h.
3. Following transfer, equilibrate the blot twice (for 5 min each time) in 100 mL of borate buffer. Use an orbital shaker at about 75 rpm.

4. Incubate the blots for 10 min in a staining solution containing 40 mL of borate buffer and 0.2 mL of the concentrated stock (35 m*M*) of MDPF in DMSO (*see* **Note 1**); use an orbital shaker (~75 rpm). The plastic box containing the blot should be covered with aluminum foil during staining. The staining conditions compatible with N-terminal sequencing of selected bands are indicated in **Note 2**.
5. After staining, rinse the blot briefly (for about 10 s) with borate buffer.
6. Place the wet membrane (*see* **Note 3**) on the UV transilluminator. Focus the camera (Polaroid or charge coupled device [CCD]) with the help of lateral illumination with a white lamp, place the optical filters indicated in **Subheading 2.7.** (*see* **Note 4**) in front of the camera lens and, in the dark, turn on the transilluminator and photograph the blot (*see* **Note 5**). Finally, turn off the transilluminator.
7. Develop the Polaroid film for the time indicated by the manufacturer (*see* **Note 6**). The images obtained with the CCD camera are stored directly in the computer and can be printed afterwards (*see* **Note 7**).
8. After photography, if specific bands have to be immunodetected (*see* **Note 8**), equilibrate the stained blot for 15 min in phosphate buffered saline containing 0.1% Tween 20, and then perform the ECL immunodetection according to the manufacturer's instructions. An example of immunodetection with the ECL system (*see* **Subheading 2.8.**) after MDPF staining is presented in **Fig. 2B.**
9. Mark with a soft pencil the stained protein bands (*see* **Note 2**) to be sequenced (*see* **Note 9**). Use a UV transilluminator to visualize the fluorescent bands. Cut out of the membrane the selected bands and apply them to the sequencer (we have used an LF3000 Automatic Sequencer [Beckman, Palo Alto, CA], *see* **Ref. *1***).

4. Notes

1. The reaction of MDPF with different proteins in solutions containing 10 m*M* sodium borate, pH 9.5, is completed in <10 min ***(4)***.
2. When the protein bands have to be used for sequencing (*see* **Note 9**), use a lower concentration of MDPF (0.1 mL of the concentrated solution of MDPF in 100 mL of borate buffer) and reduce the incubation time to 2 min. The sensitivity obtained under these conditions is lower than that obtained using the normal staining conditions (*see* **Note 5**), but it is high enough to detect bands containing protein amounts suitable for microsequencing by Edman degradation.
3. Membranes must be wet during visualization and imaging. Wet membranes are highly translucent, but dry membranes are opaque and reduce dramatically the sensitivity ***(1)***.
4. Wratten filters nos. 3 and 47 allow the visualization of the blue fluorescence emission from MDPF-labeled bands. In addition, these filters eliminate the light coming from the transilluminator and the background fluorescence produced by Nile red adsorbed on the surface of the PVDF membrane during the electrotransfer.

5. Electrophoretic bands containing 5–10 ng of protein can be seen in the images obtained with Polaroid film and the CCD system. However, considering that the yield of protein transfer from the gel to the membrane is relatively low, the actual sensitivity is presumably higher. In fact, we have observed that when proteins are transferred directly to the membrane using a slot-blotting device, 0.5 ng of protein per slot can be detected *(1)*.
6. The development time of Polaroid film is dependent on the film temperature. Store the film at 4°C (*see* **Subheading 2.7.**), but allow them to equilibrate at room temperature before use.
7. Ink jet printers with glossy paper yield images with photographic quality (*see* e.g., **Fig. 2A**).
8. Our results *(1)* have shown that the covalent modification of blotted proteins produced by the reaction with MDPF does not alter the antigenic properties that allow the binding of polyclonal antibodies.
9. Our results *(1)* have shown that the staining conditions indicated in **Note 2** do not preclude further sequencing reactions with a high yield. Since MDPF reacts with primary amino groups of proteins (*see* **Fig. 1**), probably including N-teminal groups, these results indicate that under these staining conditions the reaction with MDPF is not complete.

Acknowledgment

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Copper Iodide Staining of Proteins and Its Silver Enhancement

Douglas D. Root and Kuan Wang

1. Introduction

Copper iodide staining and silver-enhancement is designed to quantify proteins adsorbed to solid surfaces such as nitrocellulose, nylon, polyvinylidene difluoride (PVDF), silica, cellulose, and polystyrene ***(1–5)*** and has important applications in Western blotting and thin layer chromatography ***(3,6)***. The binding of cupric ions to the backbone of proteins under alkaline conditions and their reduction to the cuprous state is the basis of several protein assays in solution including the biuret, Lowry, and bicinchoninic acid methods (***1–3,7*** and *see* Chapters 2–4). In the case of copper iodide staining, the protein binds copper iodide under highly alkaline conditions. This protein assay demonstrates sensitivity, speed, reversibility, low cost, and the lack of known interfering substances (including nucleic acid; **refs.** ***4,5***). Copper iodide staining is sufficiently sensitive to permit the quantification of proteins adsorbed to microtiter plates ***(5)***. The information is particularly useful for the quantitative interpretation of enzyme-linked immunosorbent assay (ELISA) and protein binding experiments. The precision of the determination of protein adsorbed to the microtiter plate by copper iodide staining is typically about 10–15%. The high sensitivity of copper iodide staining (about 40 pg/µL) may be increased several fold by a silver-enhancement procedure that allows the detection of protein down to about 10 pg/µL, which is more sensitive than common solution-based assays ***(7)***. The sensitivity of the assay can be increased by repeated applications of the protein on a membrane to concentrate it. Protein concentrations may be estimated from copper iodide staining from very dilute protein solutions or when only small amounts of a precious protein are available such as for the analysis of chromatography fractions.

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2. Materials

2.1. Copper Iodide Staining

1. Prepare the copper iodide staining reagent by mixing 12 g of $CuSO_4 \cdot 5H_2O$, 20 g of KI, and 36 g of potassium sodium tartrate with 80 mL of distilled water in a glass beaker (*see* **Note 1**). As the slurry is vigorously stirred, 10 g of solid NaOH is slowly added. The suspension becomes warmer and changes color from brown to green to dark blue. After the NaOH is completely dissolved, the beaker is allowed to cool at room temperature for 30 min without stirring to allow the brownish-red precipitate to settle. Then 70 mL of solution is aspirated from the top to leave approx 50 mL of reagent with precipitate. The reagent is stable and may be stored in a sealed bottle at room temperature for at least 1 mo or at 4°C for at least 1 yr (*see* **Note 2**).
2. Prepare the copper iodide stain remover solution with 0.19 g of $Na_4EDTA\ H_2O$, 0.28 g of $NaH_2PO_4 \cdot H_2O$, and 2.14 g of $Na_2HPO_4 \cdot 7H_2O$ in 100 mL of deionized water.
3. Nitrocellulose (e.g., BA85, Schleicher & Schuell, Keene, NH), PVDF (e.g., PVDF, Millipore Corporation, Bedford, MA), or nylon blotting paper (e.g., Zeta probe, Bio-Rad Laboratories, Richmond, CA; *see* **Note 3**).

2.2. Silver-Enhanced Copper Staining (SECS)

1. Prepare the silver enhancing reagent just prior to use by dissolving 0.1 g of $AgNO_3$, 0.1 g of NH_4NO_3, and 7 µL of 5% (v/v in water) β-mercaptoethanol in 100 mL of distilled water. After the other components are dissolved and immediately before use add 2.5 g of Na_2CO_3.
2. Prepare the SECS stain remover by dissolving 33.2 g of KI in 100 mL of distilled water (final concentration, 2 *M* KI).

2.3. Copper Iodide Microtiter Plate Assay

1. Polystyrene 96-well microtiter plates (e.g., Nunc Immuno-Plate, Denmark; Titertek 76-381-04, McLean, VA; or Immulon 1 and 2, Dynatech Laboratories, Chantilly, VA).
2. Nitrocellulose membranes (e.g., BA85, Schleicher & Schuell, Keene, NH).
3. Household 3-in-one lubricating oil (Boyle-Midway, New York, NY).
4. A standard single hole puncher (6 mm diameter).
5. Some form of densitometer is required such as a flatbed scanner or video camera and framegrabber with image analysis software (*see* **Note 4**).

3. Methods

3.1. Copper Iodide Staining

1. Stir the copper iodide staining reagent vigorously at room temperature immediately prior to use. The reagent should be a fine slurry.
2. Rock the copper iodide staining reagent over the dried protein blot (or Western blot) for at least 2 min (but not more than 5 min; *see* **Note 5**) as the reddish-brown bands appear on the blot.

3. Gently dip the stained blot up and down in three beakers of deionized water and then allow the blot to dry (*see* **Note 6**).
4. The stained blot then may be quantified by densitometry (*see* **Note 7**) and photographed for documentation.
5. The staining pattern is stable at room temperature for at least 1 yr.
6. If greater sensitivity is desired, the blot may be used directly in the silver-enhanced copper staining procedure (*see* **Subheading 3.2**).
7. If subsequent immunostaining on the same blot is required, destain for 15 min with gentle agitation in the copper iodide stain remover prior to immunostaining.

3.2. Silver-Enhanced Copper Staining (SECS)

1. Rock a nitrocellulose protein blot stained with copper iodide (*see* **Subheading 3.1**) for 5 min in freshly prepared silver-enhancing reagent until the bands become dark black.
2. Dip the blot in a 1-L beaker of deionized water and stored in the dark to dry (to prevent background development).
3. The blot then may be quantified by densitometry and photographed for documentation.
4. If subsequent immunostaining on the same blot is required, destain for 30 min with gentle agitation in the SECS stain remover prior to immunostaining (*see* **Note 8**).

3.3. Copper Iodide Microtiter Plate Assay

1. Adsorb the protein of interest to duplicate microtiter plates and note the volume (*V*) that was used to adsorb the protein in each well (*see* **Note 9**). One of the microtiter plates is for copper iodide staining and the other is for quantitative ELISA or binding experiments. The microtiter plate for copper iodide staining contains protein adsorbed to only a few of the wells (e.g., 4–16 wells; see the schematic diagram and **Note 10**).
2. Create a standard curve by dot blotting approx 5–100 ng of the protein of interest per 5-μL drop onto nitrocellulose paper and allow to air dry. Blanks are dotted with equal volumes of buffers.
3. Stain both the nitrocellulose paper and the microtiter plate with adsorbed protein by the copper iodide staining procedure (*see* **Subheading 3.1**).
4. Use the hole puncher to excise stained dots from the nitrocellulose membrane and place them stained side down into blank wells on the microtiter plate (*see* **Note 11**).
5. Use the hole puncher to excise blank nitrocellulose circles and place them in microtiter plate wells containing copper iodide-stained protein and also in some blank wells to determine the background density (*see* **Fig. 1**).
6. For transmittance densitometry, first add 5 μL per well of Household 3-in-one oil to make the nitrocellulose translucent, thus reducing the background. For reflectance densitometry, the bottom of the microtiter plate may be scanned directly (the stained sides of the nitrocellulose must all be face down in the wells).

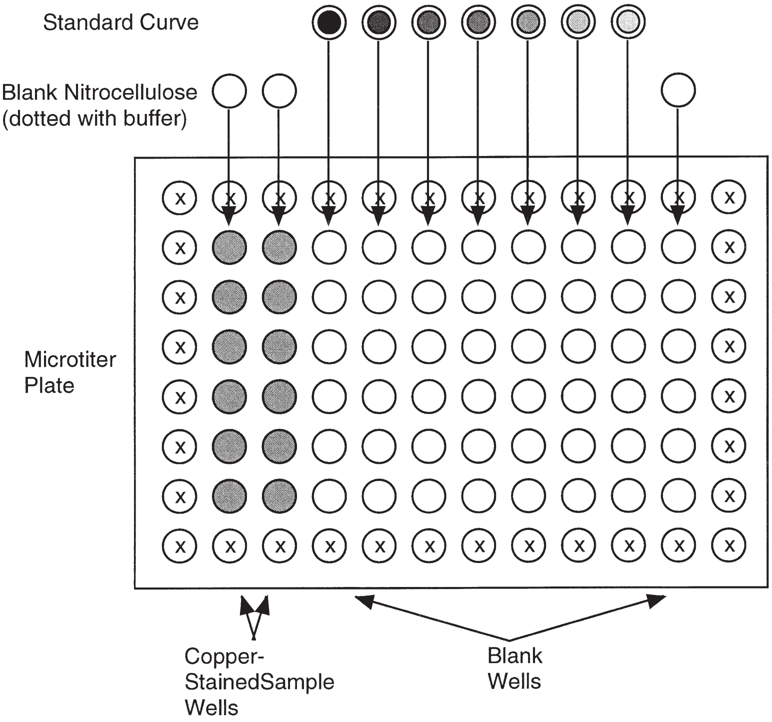

Fig. 1. Schematic diagram of the assembly of a microtiter plate for the copper iodide staining assay. Avoid wells marked with "x" due to possible distortions from edge effects.

7. Measure the mean optical density and total area of the known amounts of stained protein (adsorbed to nitrocellulose) and construct a standard curve of mean optical density vs ng/mm^2 of protein (*see* **Note 12**).
8. Measure the mean optical density of stained sample protein (adsorbed to microtiter plate) and compare to the standard curve to determine the concentration of protein (in ng/mm^2) on the microtiter plate well.
9. Calculate the total area of the stained sample protein (adsorbed to microtiter plate) on the microtiter plate well from the equation:

$$\text{Total Area} = 3.14r^2 + (2v/r)$$

in which r (in mm) is the radius of the cylindrical flat-bottom well, and V (in µL) is the volume that was used to adsorb protein to the well. The Total Area (typically 94.7 mm^2 for a 100-µL volume applied to a plate with a 3.25-mm well radius) multiplied by the surface density (in ng/mm^2) of the sample protein yields the amount of protein (in nonograms) adsorbed to each microtiter plate well.

4. Notes

1. Proportions of sodium and potassium ions in the copper iodide staining reagent are important. Thus potassium sodium tartrate (sodium potassium tartrate) should not be substituted with, for instance, sodium tartrate.
2. The copper iodide staining reagent can generally be reused two or three times but will eventually become less sensitive.
3. Copper iodide staining reagent stains proteins adsorbed on most solid phase adsorbents including nitrocellulose, nylon, PVDF, silica, cellulose, and polystyrene. PVDF requires that the blot be prewetted in 50% methanol immediately prior to staining, and some smearing of bands can occur at high loads of protein with PVDF. Copper iodide staining of silica leaves an uncharacteristic bluish background. Comparisons of the sensitivities of copper iodide staining and SECS with other stains on a variety of common supports are illustrated in **Table 1**.
4. Microtiter plate readers are to be avoided for quantitative measurements, because they are usually not sensitive enough (copper iodide staining yields OD ≤ 0.1) and do not sample a large enough area of the stained surface to detect any nonuniformity in the staining density.
5. Exceeding a staining time of 5 min can both damage nitrocellulose membranes and lead to solubilization of adsorbed protein. A staining time of 2 min is optimal.
6. Washing of microtiter plates should be handled gently by dipping in beakers of deionized water. Vigorous washing procedures often lead to nonuniform protein distribution and consequent uneven staining of microtiter plates.

Table 1
Comparison of the Detection Limits (in ng/1µL) of Protein Stains[a]

Stain	Nitrocellulose	PVDF	Nylon	Silica	Cellulose
SECS	0.01	0.071	1	5	0.02
Copper iodide	0.04	0.014	5	20	0.04
Kinetic silver staining[b]	40	None	300	70	0.01
Coomassie[c]	9	150	None	40	300
Eu metal chelate[d]	9	150	2000	70	150

[a] Two-fold serial dilutions of bovine albumin were dotted in 1-µL spots on the indicated support, then dried, stained, and evaluated visually.

[b] Performed as described in Chapter 8.

[c] Performed as described in **ref. *8***.

[d] Performed as described in **ref. *9***. The Eu metal chelate stain was visualized over a UVP (Upland, CA) 302 nm UV transilluminator. When spots were excised and quantified by time-resolved fluorescence in an SLM Aminco Bowman II spectrometer, a detection limit of 3 ng/1 µL was observed on nitrocellulose consistent with reported values ***(9)***.

7. An example of a low cost densitometer is a desktop flatbed scanner, color Apple Macintosh computer, and NIH Image software (public domain, by Wayne Rasband; further details are available by personal communication).
8. SECS may be removed by concentrations of KI that are less than 2 *M* but will require longer incubations (e.g., 90 min for 0.5 *M* KI).
9. The wells on the edge of microtiter plates should be avoided for quantitative measurements because they tend to yield less accurate numbers.
10. Nitrocellulose quantitatively binds most proteins that are dot blotted onto it and retains them well throughout copper iodide staining.
11. If there is a problem with static repulsion and transmission densitometry will be used, the Household 3-in-one oil (**Subheading 3.3., step 6**) may be first applied to the microtiter plate well to release the static charge.
12. Quantitative measurements of copper iodide staining should be done at least in triplicate because of the relatively high (10–15%) standard deviation of the results.

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Detection of Proteins on Blots Using Direct Blue 71

Wei-Tao Cong, Sun-Young Hwang, Li-Tai Jin, Jung-Kap Choi

1. Introduction

The visualization of proteins blotted onto transfer membranes has important applications for the localization of bands prior to further steps and for the comparison of individual proteins detected by immunostaining with total proteins ***(1,2)***. Consequently, sensitive, convenient, and quantitative total protein stained on membranes is required ***(3,4)***. Among transfer membranes available (NC, PVDF, and nylon), NC appears to be the material of choice because it is relatively inexpensive and requires a fast and simple step for blocking from nonspecific binding ***(5)***. The widely used methods for staining of NC membranes are Amido Black ***(2,6)***, Coomassie Blue ***(7,8)***, India ink ***(9,10)***, and Ponceau S ***(11,12)*** staining (*see* **Note 1**). However, none of them is as sensitive as silver stain on gels ***(4)***. Metal stains (colloidal gold stain, silver stain) of membranes can provide the highest sensitivity, but they have certain disadvantages: long incubations, troublesome preparation, an expensive metal, or irreversible protein staining ***(4,13)***. Copper iodide staining is also sensitive, but complicated steps are required to prepare reagents. Furthermore, this method employs strongly alkaline solution of pH 13.8, resulting in membrane impairment after incubation for more than 5 min ***(14)***. Recently, reversible metal complex stains were introduced, which are compatible with subsequent evaluation ***(15-17)***.

We have attempted to develop a dye-based staining method with sensitivity, convenience, and economy. DB71 (C.I. 34140) produces blue color in water with an absorption maximum at 594 nm and possesses excellent light fastness (Product Information of Organic Dyestuffs Co., East Providence, RI). No hazard has been connected with the use of the dye under normal conditions. The structure of the dye consists of four biphenyls with a hydroxyl, a primary amine, three azo, and four sulfonate groups (*see* **Fig. 1**). The relatively strong hydrophobicity

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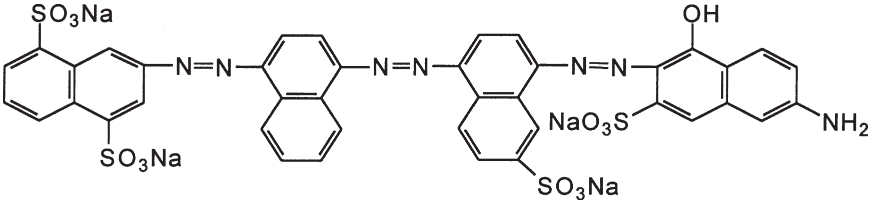

Fig. 1. Chemical structure of DB71.

Table 1
Comparison of Staining Procedures for Protein Blots[a]

Dyes	Conditions	Approx. detection limits (ng)
0.05% Coomassie Blue R-250	S: 50% MeOH–7% HAc (10 min) D: 10% MeOH–14% HAc (overnight at 65°C)	100[d]
0.1% Amido Black 10B	S: 45% MeOH–10% HAc (10 min) D: 10% HAc (15 min)	50[d]
0.1% Ponceau S	S: 5% HAc (3 min) W: Water[b]	100[d]
0.05% CPTS	S: 12 m*M* HCl (~1 min) W: 12 m*M* HCl[b]	10[d]
0.008% DB71	S: 40% EtOH–10% HAc (5 min) W: 40% EtOH–10% HAc[c]	5–10 (our work)

[a] The staining procedures of Coomassie Blue ***(9)***, Amido Black ***(6)***, and CPTS ***(21)*** were performed as in references cited. Ponceau S staining followed the product information of Sigma Chemical Co.

[b] Membranes were briefly washed in several changes of the solutions to remove excess dye.

[c] Membranes were washed once in the solution, since background was almost clear.

[d] Approximate detection limits are from the references cited, where they were determined by comparing protein slot blots stained by these methods.

S, staining; D, destaining; W, washing.

imparted by four biphenyl rings of DB71 is expected to improve the low sensitivity observed in Ponceau S staining (*see* **Notes 2** and **3; Table 1**). On the basis of this reasoning, DB71 appears to be a satisfactory candidate for protein staining on membranes (*see* **Notes 4** and **5; ref.** ***18***).

2. Materials

1. Stock dye solution: 0.1% [w/v] DB71. Dissolve 0.1 g of DB71 (dye content, 50%; from Aldrich) in 100 mL of distilled water. The stock solution is stable for months at room temperature.
2. Working dye solution: 0.008% [w/v] DB71. Dilute 4 mL of stock dye solution to 50 mL of washing solution (*see* **Note 6**). The working solution is stable for weeks at room temperature.
3. Washing solution: For 1 L of solution, mix 500 mL of distilled water with 400 mL of absolute ethanol (EtOH) and 100 mL of acetic acid (HAc) (*see* **Notes 7–9**).
4. Destaining solution: For 1 L of solution, mix 350 mL of distilled water with 500 mL of absolute EtOH and 150 mL of 1 *M* sodium bicarbonate (*see* **Note 10**).

3. Methods

3.1. Membrane Staining

1. Prior to staining, wet protein transferred membranes, if the membranes had been dried: wet NC membranes for a minute in distilled water, and wet PVDF membranes first in MeOH, then rinse them in distilled water.
2. Immerse membranes gently in working solution for 5 min (*see* **Note 11**).
3. Rinse membranes briefly with washing solution for seconds.
4. Allow membranes to be air-dry or wrap them up with Saran Wrap to stay wet (*see* **Note 12**).
5. Keep membranes in a refrigerator for further uses.

3.2. Dye Removal

1. After staining, if necessary, incubate membranes for 5–10 min in destaining solution (*see* **Note 13**).
2. Rinse membranes briefly with distilled water.
3. Proceed to next steps (*see* **Note 14**).

4. Notes

1. Both Coomassie Blue and Amido Black are inferior to DB71 stain in terms of requiring destaining solution and lengthy time (*see* **Table 1**), and giving lower sensitivity on PVDF. While DB71 staining is based on dye binding to proteins under acidic conditions similar to that in Amido Black ***(2,6)***, Coomassie Blue ***(7,8)***, and Ponceau S ***(11,12)*** staining methods.
2. The detection limits of DB71 staining for slot blot and electroblot are 5–10 ng of protein on NC, which is 10-fold more sensitive than Ponceau S staining that detected down to 50–100 ng. DB71 can also stain proteins bound to PVDF; however, background is colored a little, possibly due to the relatively increased hydrophobicity in PVDF, comparing with NC. DB71 staining detects down to 10–20 ng of protein on PVDF, whereas Ponceau S staining does to 100 ng of protein.

3. DB71 is comparable with copper phthaloryanine-3,4' ,4",4 "'-tetrasulfonic acid (CPTS) (*see* **Figs. 2C** and **G**) reported as the highest sensitive dye-binding staining method ***(19)***, in its sensitivity, rapidity, and reversibility. The bluish violet color of DB71 stained proteins gives better band contrast than the turquoise blue CPTS stained ones, allowing easy photography without any filter system; moreover, DB71 is much cheaper than CPTS.
4. DB71 possesses the properties of an ideal protein stain, including high and nonspecific affinity for protein; rapid staining; convenient application conditions compatible with matrix material and protein; large molar absorptivity with the absorption maximum in blue region; and safe to use ***(19)***.
5. It is noticeable that among the methods tested, DB71 staining allows the highest band contrast on PVDF with a similar staining intensity as on NC (*see* **Figs. 2D** and **H**).
6. At concentrations < 0.002%, the staining intensity of protein bands is weak; at concentrations > 0.01%, unwanted background coloration is obtained.
7. The binding of DB71 to proteins is favored under acidic condition: It is quite probable that in DB71 staining solution most carboxyl groups of proteins

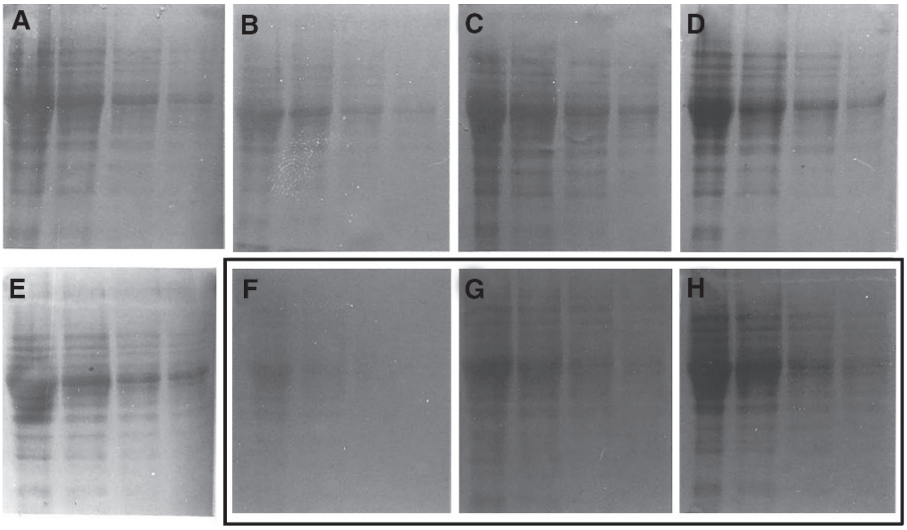

Fig. 2. Comparison of DB71 staining with various dye-based staining methods. Blots contained a whole cell lysate of HL-60, a human promyelocytic leukemia cell line, that 2×10^6 cells were lysed in 150 µL of lysis buffer. Four serial half dilutions of the lysates were separated on 10% SDS-PAGE and transferred onto two types of membranes (**A–E**, NC, **F–H**, PVDF). Transferred proteins were visualized by Coomassie Blue (**A**), Ponceau S (**B** and **F**), CPTS (**C** and **G**), DB71 (**D** and **H**), and Amido Black (**E**). All staining procedures were as described in **Table 1**.

are protonated and thus there is no electrostatic repulsion between proteins and the dye molecule containing four sulfonate groups. In the acidic solutions, ionic interactions between maximally protonated amino moieties of proteins and anionic functional groups of the dye might primarily contribute to the stain. Additional forces for the staining could involve hydrophobic interaction, hydrogen bond, and Van der Waals forces which have been reported to be the binding mode of proteins to most anionic dyes ***(20)***.

8. For the visualization of proteins in gels and membranes, most of dye-based protein staining methods have generally employed MeOH as a component of staining/destaining solution ***(2,6-9)***, despite its toxicity. DB71 can use EtOH instead of MeOH, without any loss of staining intensity by the dye.
9. DB71 staining is influenced by the concentration of EtOH. Less than 20% EtOH causes background coloration; more than 60% EtOH not only reduces band intensity, but also causes distortion of the membranes.
10. The removal of DB71 from stained bands requires incubating membranes in ethanol–1 *M* sodium bicarbonate–water (10:3:7) for a few minutes. Complete destaining, however, may take up to an hour or more, depending on the amount or nature of proteins of interest. The membranes can be repeatedly stained and destained, with no apparent loss of sensitivity.

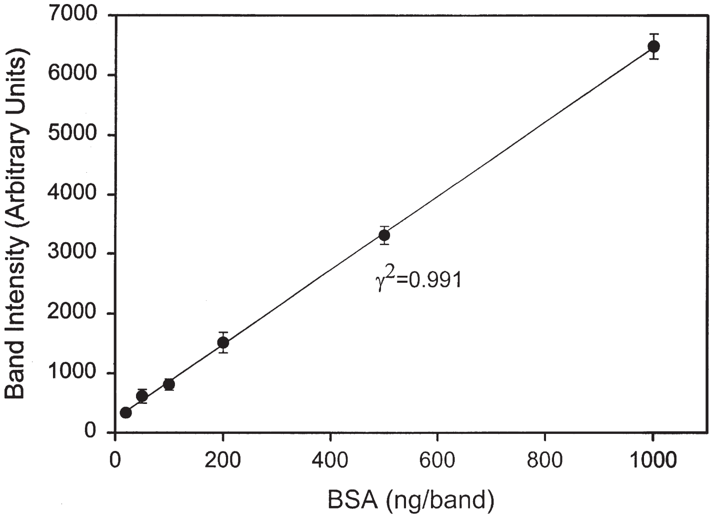

Fig. 3. Standard assay of BSA after DB71 staining. The peak areas of the densitometric measurements of BSA representing the relative band intensities were used to obtain a response curve (*see* **Note 17**). Electroblotted BSA was stained for 5 min in 0.008% DB71 solution and washed briefly with 40% EtOH–10% HAc, and quantified by using a TINA 2.09 software program. Data are expressed as the means ± SEM of the mean of triplicate of the experiments.

11. In DB71 staining solution containing 0.008% DB71 in 40% EtOH–10% HAc, major protein bands appear in usually 1–2 min, with minor protein bands being detected within 5 min.
12. A higher band contrast for photography is obtained when the stained membrane keeps wet rather than dry. The stain remains stable for several months in a refrigerator.
13. DB71 staining is applicable to a subsequent immunostaining without removing the dye from developed bands. However, we recommend the dye removal, prior to the immunodetection of small amounts of a particular protein.
14. DB71 staining can also be applied for quantification purposes with simplicity and convenience over the conventional spectrophotometric procedures. The relationships between peak area representing band intensity after DB71 staining and the protein amounts hold a linearity between 20 and 1000 ng, with a correlation coefficient of 0.991 (*see* **Fig. 3**).

Acknowledgment

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Detection of Proteins on Western Blots Using Colorimetric and Radiometric Visualization of Secondary Ligands

Nicholas J. Kruger

1. Introduction

Western blotting (immunoblotting) provides a simple and effective method for identifying specific antigens in a complex mixture of proteins. Initially, the constituent polypeptides are separated by SDS-polyacrylamide gel electrophoresis, or a similar technique, and are then transferred either electrophoretically or by diffusion onto a nitrocellulose filter. Once immobilized onto the membrane, specific polypeptides can be identified by probing with antibodies that bind to antigens retained on the filter and subsequently visualizing the resulting antibody-antigen complex. This chapter describes conditions suitable for binding antibodies to immobilized proteins and methods for locating these antibody-antigen complexes using appropriately labelled ligands. These methods are based on those of Blake et al. ***(1)***, Burnette ***(2)*** and Towbin et al. ***(3)***.

Although there are many techniques for visualizing antibodies bound to nitrocellulose, most exploit only two different types of ligand; one is protein A and the other is an antibody raised against immunoglobulin G (or other class of immunoglobulin) from the species used to generate the primary antibody. Protein A, from the cell wall of *Staphalococcus aureus*, specifically binds the Fc region of immunoglobulin G (IgG) from many mammals ***(4)***. Thus, this compound provides a general reagent for detecting antibodies from several sources. With this ligand, as little as 0.1 ng of protein may be detected using the simple techniques described here, though the precise amount will vary with the specific antibody titer ***(5)***. The principal disadvantage of protein A is that it fails to bind effectively to major IgG sub-classes from several experimentally

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Table 1
Variation in species specificity of various immunogobulin-binding proteins

Species	Immunoglobulin class	Affinity of binding by Protein A	Protein G	Protein A/G	Protein L
Human	IgG1*	+++	+++	+++	+++
	IgG2	+++	+++	+++	+++
	IgG3	–	+++	+++	+++
	IgG4	+++	+++	+++	+++
	IgM	–	–	–	+++
	IgA	–	–	–	+++
	IgE	–	–	–	+++
	IgD	–	–	–	+++
Mouse	IgG1*	+	++	++	+++
	IgG2a	+++	+++	+++	+++
	IgG2b	++	++	+++	+++
	IgG3	+	++	+++	+++
Rat	IgG1	–	++	++	+++
	IgG2a	–	+++	+++	+++
	IgG2b	–	+	+	+++
	IgG2c	+	+++	+++	+++
Cow	IgG	++	+++	+++	–
Cat	IgG	+++	–	+++	n.d.
Chicken	IgG	–	+	+	++
Dog	IgG	+++	+	+++	+
Goat	IgG	+/–	++	+++	–
Guinea pig	IgG	+++	+	+++	++
Hamster	IgG	+	++	++	+++
Horse	IgG	+/–	–/+++	+++	+/–
Pig	IgG	+++	+	+++	+++
Rabbit	IgG	+++	++	+++	+
Sheep	IgG	+/–	+++	+++	–

The binding affinities for immunoglobins from different sources is indicated as follows: –, no binding; +, low; ++, moderate; +++, high; n.d., not determined. * Denotes major sub-class of IgG. Complied from information provided by Pierce Co. (technical resource TR0034.1) and data in references ***4, 9-11*** and references therein.

important sources, such as rat, mouse, goat and sheep (*see* **Table 1**). For antibodies raised in such animals a similar method using derivatives of other bacterial immunoglobulin-binding proteins may be suitable (*see* **Note 1**). Alternatively, antibody-antigen complex bound to the nitrocellulose filter may be detected using a secondary antibody raised against IgG from the species used to generate the primary antibody. An advantage of such secondary antibody systems is that they bind only to antibodies from an individual species, and this specificity may be exploited to identify multiple polypeptides on a single nitrocellulose membrane (***6,7***).

Strategies for visualizing the ligand bound to the antibody-antigen complex on the membrane generally fall into three broad categories. One is direct detection, in which the ligand itself is tagged with some readily detectable marker, typically a radioactive label such as ^{125}I, or gold particles or a fluorescent dye. The second is indirect detection, in which the ligand is linked to a marker enzyme and visualization is dependent on localizing a product of the reaction catalysed by the enzyme activity. The third is a hybrid strategy in which the ligand is conjugated to biotin and this, in turn, is detected using a marker enzyme linked to avidin (or streptavidin).

The marker enzymes most commonly used for detection are alkaline phosphatase and horseradish peroxidase. Both can be linked efficiently to other proteins, such as antibodies, protein A and avidin, without interfering with the function of the latter proteins or inactivating the enzyme. Moreover, a broad range of synthetic substrates have been developed for each of these enzymes. Enzyme activity is normally visualised by incubating the membrane with an appropriate chromogenic substrate which is converted to a colored, insoluble product. The latter precipitates onto the membrane in the area of enzyme activity, thus identifying the site of the antibody-antigen complex (*see* **Note 2**).

Both antigens and antisera can be screened efficiently by western blotting. Probing of a crude extract after fractionation by SDS-polyacrylamide gel electrophoresis can be used to assess the specificity of an antiserum. The identity of the antigen can be confirmed using a complementary technique, such as immunoprecipitation of enzyme activity. This information is essential if the antibodies are to be used reliably. Once characterized, an antiserum may be used to identify antigenically related proteins in other extracts using the same technique. Examples of the utility and application of western blotting have been reviewed by Kurien and Scofield ***(8)***.

2. Materials

1. Electrophoretic blotting system, such as Trans-Blot or Mini Trans-Blot supplied by Bio-Rad.
2. Nitrocellulose paper: 0.45 µm pore size (*see* **Note 3**).
3. Protein A derivatives.
   a) Alkaline phosphatase-conjugated protein A obtained from Sigma-Aldrich Co. Dissolve 0.1 mg in 1 mL of 50% (v/v) glycerol in water. Store at −20°C.
   b) Horseradish peroxidase-conjugated protein A obtained from Sigma-Aldrich Co. Dissolve 0.1 mg in 1 mL of 50% (v/v) glycerol in water. Store at −20°C.
   c) ^{125}I-labelled protein A, specific activity 30 mCi/mg. Affinity-purified protein A, suitable for blotting, is available commercially (*see* **Note 4**). **^{125}I emits**

γ-radiation. Be sure that you are familiar with local procedures for safe handling and disposal of this radioisotope.

4. Secondary antibody: A wide range of both alkaline phosphatase and horseradish peroxidase conjugated antibodies are available commercially. They are usually supplied as an aqueous solution containing protein stabilizers. The solution should be stored under the conditions recommended by the supplier. *Ensure that the enzyme-linked antibody is against IgG of the species in which the primary antibody was raised.*
5. Washing solutions: Phosphate buffered saline (PBS). Make 2 L containing 10 m*M* NaH_2PO_4, 150 m*M* NaCl adjusted to pH 7.2 using NaOH. This solution is stable and may be stored at 4°C. It is susceptible to microbial contamination, however, and is usually made as required. The other washing solutions are made by dissolving the appropriate weight of bovine serum albumin or Triton X-100 in PBS. Dissolve Bovine serum albumin by rocking the mixture gently in a large, sealed bottle to avoid excessive foaming. The “blocking” and “antibody” solutions containing 8% albumin may be stored at −20°C and reused several times. Microbial contamination can be limited by filter-sterilizing these solutions after use or by adding 0.05% (w/v) NaN_3 (*but see* **Note 5**). Other solutions are made as required and discarded after use.
6. Alkaline phosphatase substrate mixture:

   a) Diethanolamine buffer. Make up 100 m*M* diethanolamine and adjust to pH 9.8 using HCl. This buffer is usually made up as required, but may be stored at 4°C if care is taken to avoid microbial contamination.

   b) 1 *M* $MgCl_2$. This can be stored at 4°C.

   Combine 200 µL of 1 *M* $MgCl_2$, 5 mg nitroblue tetrazolium, 2.5 mg 5-bromo-4-chloroindolyl phosphate (disodium salt, *see* **Note 6**). Adjust the volume to 50 mL using 100 m*M* diethanolamine buffer. Make up this reaction mixture as required and protect from the light before use.
7. Horseradish peroxidase substrate mixture:

   a) Make up 50 m*M* acetic acid and adjust to pH 5.0 using NaOH. This buffer is usually made up as required, but may be stored at 4°C if care is taken to avoid microbial contamination.

   b) Diaminobenzidine stock solution of 1 mg/mL^{-1} dissolved in acetone. Store in the dark at −20°C. **Caution: Diaminobenzidine is a potential carcinogen; handle with care.**

   c) Hydrogen peroxide at a concentration of 30% (v/v). This compound decomposes, even when stored at 4°C. The precise concentration of the stock solution can be determined by measuring its absorbance at 240 nm. The molar extinction coefficient for H_2O_2 is 43.6 M^{-1}.cm^{-1} at this wavelength (*see* **Note 7**).

   Combine 50 mL of acetate buffer, 2 mL of diaminobenzidine stock solution and 30 µL of hydrogen peroxide immediately before use. Mix gently and avoid vigorous shaking to prevent unwanted oxidation of the substrate. Protect the solution from the light.
8. Protein staining solutions: These are stable at room temperature for several weeks and the stains may be reused.

a) Amido black stain (100mL): 0.1% (w/v) amido black in 25% (v/v) propan-2-ol, 10% (v/v) acetic acid.
b) Amido black destain (400 mL): 25% (v/v) propan-2-ol, 10% (v/v) acetic acid.
c) Ponceau S stain (100 mL): 0.2% (w/v) Ponceau S, 10% (w/v) acetic acid.
d) Ponceau S destain (400 mL): distilled water.

3. Methods

3.1. Immunodetection of Polypeptides

1. Following SDS-polyacrylamide gel electrophoresis, electroblot the polypeptides from the gel onto nitrocellulose at 50 V for 3 h using a Bio-Rad Trans-Blot apparatus, or at 100 V for 1 h using a Bio-Rad Mini Trans-Blot system (*see* **Note 8**).
2. After blotting, transfer the nitrocellulose filters individually to plastic trays for the subsequent incubations. Ensure that the nitrocellulose surface that was closest to the gel is uppermost. Do not allow the filter to dry out, since this often increases non-specific binding and results in heavy, uneven background staining. The nitrocellulose filter should be handled sparingly to prevent contamination by grease or foreign proteins. Always wear disposable powder-free examination gloves, and only touch the edges of the filter.
3. If desired, stain the blot for total protein using Ponceau S as described in **Subheading 3.3.2** (*see* **Note 9**).
4. Rinse the nitrocellulose briefly with 100 mL of PBS. Then incubate the blot at room temperature with the following solutions, shaking gently (*see* **Note 10**).
   a) 50 mL of PBS/8% bovine serum albumin for 30 min. This blocks the remaining protein-binding sites on the nitrocellulose (*see* **Note 11**).
   b) 50 mL of PBS/8% bovine serum albumin containing 50–500 µL of antiserum for 2–16 h (*see* **Note 12**).
   c) Wash the nitrocellulose at least five times, each time using 100 mL of PBS for 15 min, to remove unbound antibodies.
   d) 50 mL of PBS/4% bovine serum albumin containing an appropriate ligand for 2 h (*see* **Note 13**). This is likely to be one of the following:
      (i) enzyme-conjugated secondary antibody at the manufactures recommended dilution (normally between 1:1000 and 1:10,000)
      (ii) 5 µg enzyme-conjugated protein A
      (iii) 1 µCi ^{125}I-labelled protein A
   e) Wash the nitrocellulose at least five times, each time using 100 mL PBS/1% Triton X-100 for 5 min, to remove unbound protein A or secondary antibody. To ensure effective washing of the filter, pour off the solution from the same corner of the tray each time and replace the lid in the same orientation.

3.2. Visualization of Antigen-Antibody Complex

3.2.1. Alkaline phosphatase-conjugated ligand

In this method the enzyme hydrolyzes 5-bromo-4-chloroindolyl phosphate to the corresponding indoxyl compound. The product tautomerizes to a ketone, oxidizes, and then dimerizes to form an insoluble blue indigo that is deposited on the filter. Hydrogen ions released during the dimerization reduce nitroblue tetrazolium to the corresponding diformazan. The latter compound is an insoluble intense purple compound that is deposited alongside the indigo, enhancing the initial signal.

1. Briefly rinse the filter twice, each time using 50 mL of diethanolamine buffer.
2. Incubate the filter with 50 mL of alkaline phosphatase substrate mixture until the blue-purple products appear, usually after 5–30 min.
3. Prevent further color development by removing the substrate mixture and washing the filter thrice, each time in 100 mL of distilled water. Finally, dry the filter thoroughly before storing (*see* **Note 14**).

3.2.2. Horseradish peroxidase-conjugated ligand

Peroxidase catalyses the transfer of hydrogen from a wide range of hydrogen donors to H_2O_2, and it is usually measured indirectly by the oxidation of the second substrate. In this method, soluble 3,3´-diaminobenzidine is converted to a red-brown insoluble complex that is deposited on the filter. The sensitivity of this technique may be increased up to 100-fold by intensifying the diaminobenzidine-based products using a combination of cobalt and nickel salts that produce a dense black precipitate (*see* **Note 15**).

1. Briefly rinse the filter twice, each time using 50 mL of sodium acetate buffer.
2. Incubate the filter with 50 mL of horseradish peroxidase substrate mixture until the red-brown insoluble products accumulate. Reaction times longer than about 30 min are unlikely to be effective due to substrate-inactivation of peroxidase (*see* **Note 7**).
3. When sufficient color has developed, remove the substrate mixture and wash the filter three times with 100 mL of distilled water. Then dry the filter and store it in the dark (*see* **Note 14**).

3.2.3. ^{125}I-labelled protein A

1. If desired, stain the blot for total protein as described in **Subheading 3.3.**
2. Allow the filter to dry. Do not use excessive heat since nitrocellulose is potentially explosive when dry.
3. Mark the nitrocellulose with radioactive ink to allow alignment with exposed and developed X-ray film (*see* **Note 16**).
4. Fluorograph the blot using suitable X-ray film and intensifying screens. Expose the film at −70°C for 6–72 h, depending on the intensity of the signal.

3.3. Staining of Total Protein

Either of the following stains is suitable for visualizing polypeptides after transfer onto nitrocellulose. Each can detect bands containing about 1 µg of protein. Coomassie brilliant blue is unsuitable for nitrocellulose membranes, since generally it produces heavy background staining (*see* **Note 17**).

3.3.1. Amido Black

Incubate the filter for 2–5 s in 100 mL of stain solution. Transfer immediately to 100 mL of destain solution, and wash with several changes to remove excess dye. Unacceptably dark backgrounds are produced by longer incubation times in the stain solution.

3.3.2. Ponceau S

Incubate the filter with 100 mL of Ponceau S stain solution for 30 min. Wash excess dye off the filter by rinsing in several changes of distilled water. The proteins may be destained by washing the filter in PBS (*see* **Note 9**).

4. Notes

1. Protein G, a cell wall component of group C and group G streptococci, binds to the Fc region of IgG from a wider range of species than that recognised by protein A ***(9)***. Therefore antibodies that react poorly with protein A, particularly those from rat, mouse, goat and sheep, may be detected by a similar method using protein G derivatives. Natural protein G also contains albumin binding sites and membrane binding regions which can lead to non-specific staining. However, these problems can be avoided by using protein G', a recombinant, truncated form of the protein which lacks both albumin and membrane binding sites and is thus more specific for IgG than the native form of the protein. This is the form of protein G provided by suppliers such as Sigma-Aldrich Co.

   An alternative is protein A/G which is a recombinant fusion protein that contains four Fc binding domains from protein A and two from protein G ***(10)***. Protein A/G therefore combines the IgG binding characteristics of protein A and protein G, and as a consequence individual IgG subclasses of mouse monoclonal antibodies are likely to have a stronger affinity for the chimeric protein A/G than for either protein A or protein G ***(10)***. Protein A/G has the additional advantage of being less dependent upon pH than protein A.

   Another useful ligand is protein L from *Peptostreptococcus magus* which has affinity for kappa light chains from various species, and will bind to IgG, IgA and IgM as well as Fab, $F(ab')_2$ and recombinant scFv fragments that contain kappa light chains. It will also bind chicken IgG. However, species such as cow, goat, sheep and horse whose immunoglobulins contain predominantly lambda chains will not bind well, if at all, to protein L ***(11)***. This selectivity may be useful in some detection strategies involving mixtures of antibodies (*see* **Note 21**).

Currently, alkaline phosphatase- and horseradish peroxidase-conjugated and ^{125}I-labelled protein G are commercially available, while only the peroxidase-conjugate of protein A/G and protein L appear to be produced.

2. Several colorimetric visualization systems have been developed for both alkaline phosphatase and horseradish peroxidase. Other assay systems for these enzymes are described by Tijssen ***(4)***.
3. Nitrocellulose is the most commonly used blotting membrane but suffers from the disadvantage that it has a relatively low protein-binding capacity and is brittle when dry. The issue of mechanical strength can be avoided by using a supported nitrocellulose, such as Hybond-C Extra (GE Healthcare, formerly Amersham Biosciences) in which a polyester web has been incorporated into the membrane, making it easier to handle. Alternatively, proteins can be transferred onto a polyvinylidene difluoride (PVDF) membrane which has a higher protein-binding capacity, better physical strength and greater chemical stability. The latter property is particularly useful in allowing the membrane to be compatible with a broader range of protein analytical techniques, ranging from standard N-terminal sequencing by Edman degradation to direct determination of peptide fragments using MALDI-QIT-TOF tandem MS ***(12)***.
4. Iodination of protein A using Bolton and Hunter reagent labels the ε-NH_2 group of lysine, which apparently is not involved directly in the binding of protein A to the Fc region of IgG. This method is preferable to others, such as those using chloramine T or iodogen, which label tyrosine. The only tyrosine residues in protein A are associated with Fc binding sites, and their iodination may reduce the affinity of protein A for IgG ***(13)***.
5. Some workers include up to 0.05% (w/v) sodium azide in the antibody and washing buffers to prevent microbial contamination. However, azide inhibits horseradish peroxidase. Therefore, do not use buffers containing azide when using this enzyme.
6. In the original description of this protocol 5-bromo-4-chloroindolyl phosphate was made up as a stock solution in dimethylformamide. However, this is not necessary if the disodium salt is used since this compound dissolves readily in aqueous buffers.
7. Urea peroxide (carbamide peroxide) may be used instead of hydrogen peroxide as a substrate for peroxidase. The problems of instability, enzyme inactivation and possibility of caustic burns associated with hydrogen peroxide are reduced by using urea peroxide, which is a hydrogen peroxide-urea adduct, obtainable as a powder or tablets. A 10% (w/v) stock solution of urea peroxide is stable for several months and is used at a final concentration of 0.1% in the peroxidase substrate mixture.

8. Extra care should be taken when working with small proteins and peptide fragments below about 15 kDa. These tend to move through nitrocellulose (and PVDF) membranes during the electro-transfer step (a phenomenon often referred to as "blow-through") and their weak binding to the membrane can result in further loss during the subsequent washing steps in the blotting procedure. The losses during the processing of blots can be partly avoided by treating the membrane with glutaraldehyde prior to blocking and detection, but this is not particularly effective for peptides smaller than 6–10 kDa and does not overcome the problem of blow-through during transfer from the gel. The retention of such small proteins and peptides may be significantly improved by transfer onto metal nanoparticle-coated PVDF membranes that are generated by soaking the membrane in a solution of gold or silver nanoparticles prior to blotting ***(14)***. This approach exploits the high affinity binding of both gold and silver to protein and facilitates the effective retention of peptides as small as 2 kDa on the membrane allowing their detection at the same level of sensitivity as that of higher molecular weight proteins in the 30–60 kDa range.
9. If desired, the nitrocellulose filter may be stained with Ponceau S immediately after electroblotting. This staining apparently does not affect the subsequent immunodetection of polypeptides, if the filter is thoroughly destained using PBS before incubation with the antiserum. In addition to confirming that the polypeptides have been transferred successfully onto the filter, initial staining allows tracks from gels to be separated precisely and probed individually. This is useful when screening several antisera.
10. High background signals arising from non-specific binding of antibodies and ligands is a common problem in immunoblotting. Several factors are important in reducing the resulting background, and even relatively minor modifications to the procedure can have marked effects (***15,16***)

    First, the filter is washed in the presence of an "inert" protein to block the unoccupied binding sites. Bovine serum albumin is a commonly used protein, but others such as fetal calf serum, haemoglobin, gelatin, and non-fat dried milk have been used successfully. Economically, the latter two alternatives are particularly attractive, and in many circumstances we now use 5% (w/v) dried milk instead of bovine serum albumin.

    The quality of protein used for blocking is important, since minor contaminants may interfere with either antigen-antibody interactions or the binding of protein A to IgG. These contaminants may vary between preparations and can be sufficient to inhibit completely the detection of specific polypeptides. In this context it should be noted that milk contains varying amounts of phosphoproteins that may interfere with immunodetection using anti-phosphoserine or anti-phosphotyrosine antibodies. Milk also contains variable amounts of biotin so it should not be used with avidin/biotin-based detection systems.

For routine analysis we use bovine serum albumin (Cohn fraction V) from Sigma-Aldrich (product number A4503) or "Marvel" non-fat dried milk produced by Premier Brands UK. It is likely that similar products from other sources are equally effective. However, the suitability of individual batches of protein as a blocking agent should be checked using antisera known to react well on immunoblots.

Secondly, the background may be reduced further by including non-ionic detergents in the appropriate solutions. These presumably decrease the hydrophobic interactions between antibodies and the nitrocellulose filter. Tween 20, Triton X-100, and Nonidet P-40 at concentrations of 0.1–1.0% have been used. In my experience, such detergents may supplement the blocking agents described above, but cannot substitute for these proteins. In addition, these detergents sometimes remove proteins from nitrocellulose (*see* **Note 11**).

Thirdly, the nitrocellulose must be washed effectively to limit non-specific binding. For this, the volumes of the washing solutions should be sufficient to flow gently over the surface of the filter during shaking. The method described in this chapter is suitable for 12 × 7 cm filters incubated in 14 × 9 cm trays. If the size of the filter is significantly different, the volumes of the washing solutions should be adjusted accordingly.

Finally, decreasing the incubation temperature to 4°C may greatly decrease the extent of non-specific background binding ***(17)***.

11. Protein desorption from the membrane during the blocking step and subsequent incubations can result in the loss of antigen and decrease the sensitivity of detection ***(18)***. In some instances, this problem may be reduced by incubating the membrane in 0.1 *M* phosphate buffer (pH 2.0) for 30 min and then rinsing in PBS prior to treatment with the blocking agent. Such acid treatment is particularly effective when using non-denaturing gel blots, or SDS-PAGE blots transferred onto PVDF rather than nitrocellulose membrane ***(19)***.

    Alternatively, polyvinyl alcohol may be used as a blocking agent ***(20)***. In comparative tests, PBS containing 1 μg/mL polyvinyl alcohol produced lower background staining than other commonly used blocking agents. Moreover, the blocking effect of polyvinyl alcohol is virtually instantaneous allowing the incubation time to be reduced to 1 min and decreasing the opportunity for loss of protein from the membrane ***(20)***.

12. The exact amount of antibody to use will depend largely on its titer. Generally it is better to begin by using a small amount of antiserum. Excessive

quantities of serum tend to increase the background rather than improve the sensitivity of the technique. Non-specific binding can often be reduced by decreasing the amount of antibody used to probe the filter. Also, *see* **Notes 18** and **19**.

13. The decision as to which secondary ligand to use will be influenced by the nature of the primary antibody, the preferred visualization strategy and the availability of the relevant conjugated or tagged ligand. For most general purposes colorimetric visualization of enzyme activity provides a simple, rapid and robust technique that is equally applicable to protein A and secondary antibody conjugates. A range of colorimetric assay systems are available for both alkaline phosphatase and horseradish peroxidase ***(4)***. However, comparison between the two enzymes is difficult because the reported sensitivity limits of the assays vary considerably and most studies use different antigens, different primary antibodies and different protocols. Despite these uncertainties, alkaline phosphatase is generally considered more sensitive than horseradish peroxidase when visualized using the standard colorimetric detection systems described in this chapter. For routine work I prefer to use alkaline phosphatase-conjugated protein A.

    The disadvantage of these colorimetric assay systems is that they are relatively insensitive and are difficult to use to quantify the amount of antigen (*see* **Note 20**). However, chemiluminescent detection systems based on light emission arising from the enzyme-catalyzed degradation of synthetic substrates are available for both alkaline phosphatase and peroxidase. These permit detection using X-ray film similar to radiolabeled probes and are not only more sensitive, but also allow quantitation of antigen by densometric scanning of the exposed X-ray film (but *see* **Note 20**). The major disadvantage of this approach is that the systems are more sensitive to interference from contaminants and place greater demands on the purity of the assay components. However, quality-control detection kits are available from several manufactures such as GE Healthcare, Pierce and Bio-Rad. More recently, chemifluorescent detection systems in which enzyme activity results in the generation of a fluorescent product have been developed commercially for both marker enzymes. These are reported to improve sensitivity of detection relative to standard chemiluminescent systems, but again they are sensitive to interference and demand greater technical care. In addition, this detection system requires an appropriate fluorescence imaging system or a CCD camera coupled with a suitable excitation light source to capture the light output. However, this obviates the need for the film output to be digitized since the fluorescence emission is recorded directly.

    Despite improvements in the sensitivity of analysis of enzyme-conjugated ligands, direct detection using ^{125}I-labelled protein A remains competitive in terms of sensitivity, robustness and ease of application, and the method outlined in this article continues to be the technique of choice in critical studies. Nevertheless, many researchers would prefer to use non-radioactive methods and continuing effort is being focused on the development of fluorescence-based direct detection systems. These involve the conjugation of secondary antibodies to fluorescent organic dyes ***(21)*** or quantum dots (***22,23***). The latter are semiconductor nanoparticles (2–10 nm diameter) that absorb light over a broad spectral range and fluoresce at defined tunable wavelengths determined by their physical size ***(24)***. They are particularly attractive because of their high fluorescence yield, resistance to photobleaching, and superior dynamic range of detection. The defined emission spectra of these fluorophores enable precise visualization of specific ligands and offer the potential for simultaneous quantitation of two or more signals on a single western blot (*see* **Note 21**). However, as with chemifluorescent systems, visualization requires an appropriate fluorescence imaging system and because of the sensitivity of the detection technique care is needed to avoid interference from fluorescence artifacts. Antibodies linked directly to cyanine or other dyes are currently available from GE Healthcare and Pierce, while a range of antibodies conjugated to quantum dots as original described by QDot Corp ***(7)*** are produced by Invitrogen and Evident Technologies. Several of these ligands are marketed in kits that are designed to be "plug-and-play" substitutes for colorimetric or chemiluminescent detection systems.

14. The products of the peroxidase reaction are susceptible to photobleaching and fading. Consequently, the developed filters should be stored in the dark, and the results photographed as soon as possible. The products of the phosphatase reaction are reportedly stable in the light. However, I treat such filters in the same way - just in case!
15. To increase sensitivity of the diaminobenzidine-based staining protocol replace the standard substrate mixture with the following intensifying solution ***(25)***. Dissolve 100 mg diaminobenzidine in 100 ml of 200 mM phosphate buffer (pH 7.3). To this solution add, dropwise and with constant stirring, 5 mL of 1% (w/v) cobalt chloride followed by 4 mL of 1% (w/v) nickel ammonium sulfate. Finally add 60 µL of 30% (v/v) hydrogen peroxide just before use.
16. Alternatively, luminescent markers, such as Glogos II supplied by Stratagene, can be used for accurately marking and aligning an autoradiograph. These luminescent peel-off stickers are easily affixed to plastic wrap or paper, and emit low-level luminescence rather than radioactivity, making them a non-hazardous substitute for radioactive ink. Exposure of the marker to ambient room light for 1 minute provides sufficient luminescence to be readily detected in autoradiograph exposures lasting 45

minutes. Because most light decay occurs within the first hour, the signal produced by the marker is similar irrespective of whether the autoradiograph is exposed for 1 hour or for several days. The use of an intensifying screen increases the intensity of the image by two- to threefold, although it also decreases the resolution of the image slightly.

17. Coomassie brilliant blue R-250 should be used in preference to Ponceau S to stain proteins blotted onto PVDF since it is more sensitive and does not bind to this membrane, whereas Ponceau S appears to interact only weakly with polypeptides attached to this matrix. If greater sensitivity is required, as little as 2 ng of a polypeptide can be detected using Sypro Ruby, a metal chelate stain containing ruthenium as part of an organic complex (developed by Molecular Probes), which works well on both nitrocellulose and PVDF membranes ***(26)***. The dual-excitation maxima displayed by the ruthenium complex means that this dye exhibits luminescence upon excitation with either UV or visible light and is therefore easily visualized using a range of instruments. The main disadvantage of this dye is that the protein staining is not reversible and while it is compatible with colorimetric immunodetection of proteins on blots it may interfere with their subsequent visualization. Sypro Rose Plus, an europium-based metal chealate stain, has a similar sensitivity to Sypro Ruby and has the added advantage of being fully reversible ***(27)***. However, the stain has a single excitation maximum at 350 nm which precludes it from being visualized using visible-wavelength excitation sources. [Note that Sypro Red and Orange, which have been developed to stain proteins within polyacrylamide gels, react relatively poorly with polypeptides attached to PVDF.] An attractive alternative is MemCode, a copper-containing stain (developed by Pierce). Although slightly less sensitive than the Sypro dyes with a reported detection limit of about 25 ng, MemCode is fully reversible, compatible with both nitrocellulose and PVDF membranes, and is easily visualized as a turquoise blue stain under visible light ***(28)***.
18. Particular care should be taken when attempting to detect antigens on nitrocellulose using monoclonal antibodies. Certain cell lines may produce antibodies that recognize epitopes that are denatured by detergent. Such “conformational” antibodies may not bind to the antigen after SDS-polyacrylamide gel electrophoresis.
19. Even before immunization, serum may contain antibodies, particularly against bacterial proteins. These antibodies may recognize proteins in an extract bound to the nitrocellulose filter. Therefore, when characterizing an antiserum, control filters should be incubated with an equal amount of pre-immune serum to check whether such pre-existing antibodies interfere in the immunodetection of specific proteins.

20. Quantitation of specific antigens using this technique is difficult and must be accompanied by adequate evidence that the amount of reporting ligand (protein A or secondary antibody) bound to the filter is directly related to the amount of antigen in the initial extract. This is important, since polypeptides may vary in the extent to which they are eluted from the gel and retained by the nitrocellulose (*see* **Notes 8** and **11**). Additionally, in some tissues proteins may interfere with the binding of antigen to the filter or their subsequent detection ***(29)***. Therefore, the reliability of the technique should be checked for each extract, although it is rare for this issue to be addressed.

    Perhaps the best evidence is provided by determining the recovery of a known amount of pure antigen. For this, duplicate samples are prepared, and to one is added a known amount of antigen comparable to that already present in the extract. Ideally the pure antigen should be identical to that in the extract. The recovery is calculated by comparing the antigen measured in the original and supplemented samples. Such evidence is preferable to that obtained from only measuring known amounts of pure antigen. The latter indicates the detection limits of the assay, but does not test for possible interference by other components in the extract.

    The other major problem derives from the technical difficulties associated with quantifying the measured signal and relating this to the amount of antibody bound to the filter. In this regard it is important to recognise that the colorimetric detection systems described in this chapter cannot be quantified reliably and to resist the temptation to use this technique to obtain anything other than the very crudest indication of relative amounts of antigen in different samples on the same blot.

    In principle, images generated on X-ray film after probing a blot with ^{125}I-labelled protein A or using a chemiluminescent detection system may be quantified by digitization on a densitometer. However, film-based detection suffers several drawbacks. First, although the film itself has a dynamic range of 5×10^2, in practice the linear range of detection for chemiluminescent systems is typically only about 10^1. In addition, film exhibits reciprocity failure. At low signal levels a threshold level of signal is required before an image is generated, and pre-flashing of the film is required to overcome this effect ***(30)***. At the other extreme, high signal levels saturate the response meaning that the darkening of the film is again not accurately recording the signal that is generated, and this can only be overcome by shortening the exposure time. Therefore when using X-ray film it is important that either different amounts of sample are analysed or several exposures are taken to ensure the signal from the blot is within the linear detection range of the film. Alternatively, many of these issues may be circumvented by using a PhosphorImager to quantify the signal ***(31)***. Similarly, quantification of signals generated by fluorescence-based visualization systems is relatively easy using a CCD camera or flat bed laser scanner which capture the image as a directly quantifiable digital signal. The wide dynamic range (10^3–10^5) of these detection systems ensure that the range over which samples can be quantified is greater than that using film. Although, in practise, the binding capacity of the membrane and steric hindrance in antibody-antigen binding usually restrict the signal detection range to 10^2–10^3, this is sufficient for the majority of biochemical studies.

21. The amount of a specific protein in a sample is usually quantified by expressing the signal intensity relative to that obtained from the same protein in a reference sample (which might be a control extract or a known amount of the pure protein). However, care must be taken if measuring the signals by autoradiography because the density of the image is not necessarily linearly related to the amount of light or radioactivity (*see* **Note 20**), and this is likely to underestimate any differences between samples ***(32)***. A refinement of this approach is to quantify the amount of the antigen of interest by measuring its signal intensity relative to that of a reference protein (typically a constitutively expressed polypeptide) in the same sample. This approach normally requires that the two (or more) proteins can be visualized separately using antibodies raised in different hosts. This is greatly facilitated by the availability of different fluorescently tagged antibodies that allow the identification of several proteins independently in a single sample in a process known as "multiplexing." This approach has the added advantage of compensating for variation between samples in the amounts of protein that are transferred to the blot.

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Identification of Glycoproteins on Nitrocellulose Membranes Using Lectin Blotting

Patricia Gravel

1. Introduction

Glycoproteins result from the covalent association of carbohydrate moieties (glycans) with proteins. The enzymatic glycosylation of proteins is a common and complex form of post-translational modification. It has been established that glycans perform important biological roles including: stabilisation of the protein structure, protection from degradation, control of protein solubility, of protein transport in cells and of protein half-life in blood. They also mediate the interactions with other macromolecules and the recognition and association with viruses, enzymes and lectins ***(1-6)***.

Carbohydrate moieties are known to play a part in several pathological processes. Alterations in protein glycosylation have been observed, for example, with the membrane glycoproteins of cancer cells, with the plasma glycoproteins of alcoholic patients and patients with liver disease, with the glycoproteins in human brains from patients with Alzeimer disease, inflammation and infection. These changes provide the basis for more sensitive and more discriminative clinical tests ***(2, 7-11)***.

Lectins are carbohydrate-binding proteins and can be used to discriminate and analyze the glycan structures of glycoproteins. The lectin-blotting technique detects glycoproteins separated by electrophoresis (SDS-PAGE or two-dimensional polyacrylamide gel electrophoresis 2-D PAGE) and transferred to membranes. This chapter describes the protocol for the detection of glycoproteins on nitrocellulose membrane using biotinylated lectins and avidin conjugated with horseradish peroxidase (HRP) or with alkaline phosphatase (AP) (*see* **Note 1**). These complexes are subsequently revealed either by a chemiluminescent or a colorimetric reaction. To illustrate this method, human plasma glycoprotein patterns, obtained after the separation of proteins by 2-D PAGE and different lectin

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incubations, are presented in **Figs. 1** to **4** (*see* **Note 2**). The principal merits of this separation technique are the high resolution and reproducibility of the separation and the high loading capacity. Using the lectin blotting technique after 2-D PAGE separation, the modification of glyproteins that affect the global charge (e.g. alteration of the sugar sialic acid) and/or the molecular weight result in multiple isoforms. Different protein alterations associated to alcohol-related disease has been identify and confirmed using this lectin blotting technique coupled to 2-D PAGE ***(12)***.

1.1. Classification of Glycoproteins

A useful classification of glycoproteins is based on the type of glycosidic linkage involved in the attachment of the carbohydrate to the peptide backbone. Three types of glycan-protein linkage have been described:

1. N-glycosidic linkage of N-acetyl glucosamine to the amide group of asparagine:

   GlcNAc (β-1,N) Asn

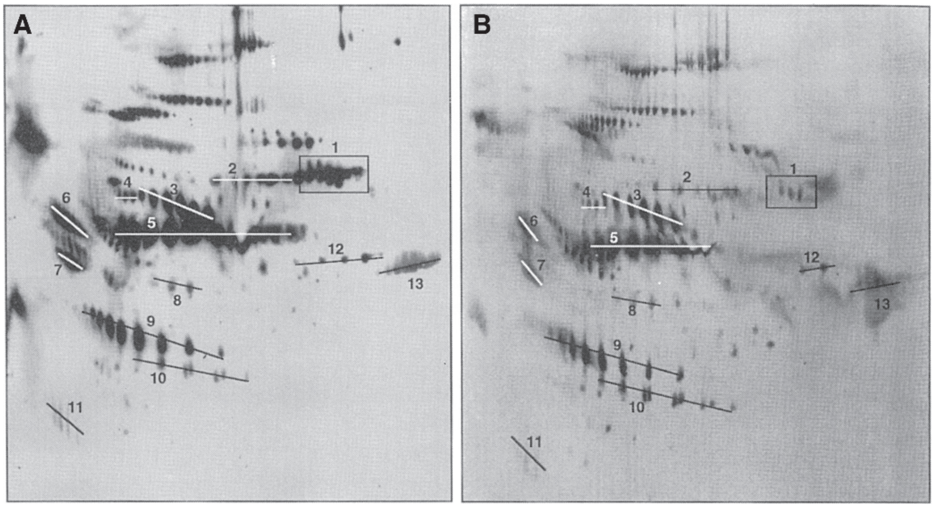


Fig. 1. Glycoprotein blot pattern of 2-D PAGE separation of plasma proteins (120 μg) probed with WGA (specific for N-acetylglucosamine and neuraminic acid) and **(A)** revealed with chemiluminesence (15 s film exposure) and **(B)** with NBT/BCIP (20 min for the development of the color reaction). Since most of the glycoproteins in plasma contain one or more N-linked glycans with at least two N-acetylglucosamine residues, the use of WGA allows a general staining of N-linked glycoproteins. 1) transferrin, 2) IgM μ-chain, 3) hemopexin, 4) α1-β-glycoprotein, 5) IgA α-chain, 6) α1-antichymotrypsin, 7) α2-HS-glycoprotein, 8) fibrinogen γ-chain, 9) haptoglobin β-chain, 10) haptoglobin cleaved β-chain, 11) apolipoprotein D, 12) fibrinogen β-chain, 13) IgG γ-chain.

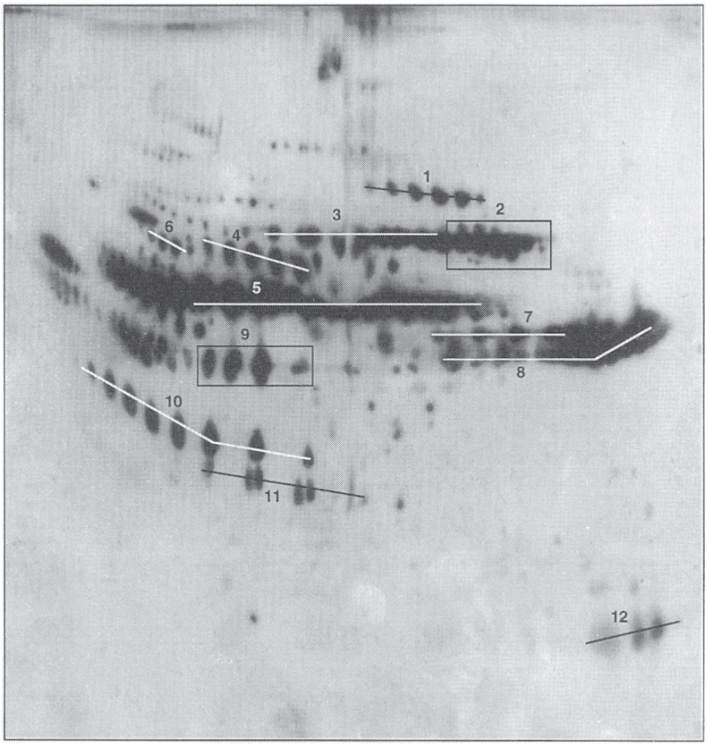


Fig. 2. Glycoprotein blot pattern of 2-D PAGE separation of plasma proteins (120 µg) probed with RCA (specific for galactose) and revealed using chemiluminescence reaction (5 s film exposure). 1) complement 3 α-chain, 2) transferrin, 3) IgM µ-chain, 4) hemopexin, 5) IgA α-chain, 6) α1-β-glycoprotein, 7) fibrinogen β-chain, 8) IgG γ-chain, 9) fibrinogen γ-chain, 10) haptoglobin β-chain, 11) haptoglobin cleaved β-chain, 12) Ig light chain.

2. O-glycosidic linkage of N-acetylgalactosamine to either the hydroxyl group of serine or threonine:

   GalNAc (α-1,3) Ser

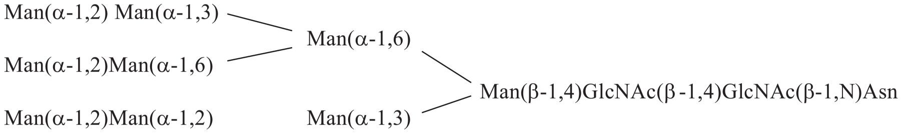
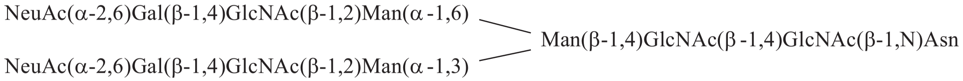
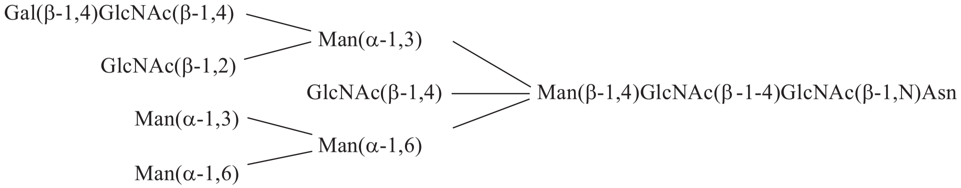
   GalNAc (α-1,3) Thr

3. O-glycosidic linkage of galactose to 5-hydroxy-lysine:

   Gal (β-1,5) OH-Lys

Most plasma glycoproteins bear exclusively asparagine-linked oligosaccharides ***(13)*** and these N-glycosidic linkages are by far the most diverse. They can be subdivided into 3 groups according to structure and common oligosaccharide sequences (**Table 1**). They all have in common the inner-core structure presented

Table 1
Glycans *N*-Glycosidically Conjugated to Proteins

Type of *N*-glycosidic linkage	Examples of structures
High mannose type	 Man(α-1,2) Man(α-1,3) Man(α-1,2)Man(α-1,6) Man(α-1,6) Man(α-1,2)Man(α-1,2) Man(α-1,3) Man(β-1,4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn
Complex type	 NeuAc(α-2,6)Gal(β-1,4)GlcNAc(β-1,2)Man(α-1,6) NeuAc(α-2,6)Gal(β-1,4)GlcNAc(β-1,2)Man(α-1,3) Man(β-1,4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn
Hybrid type	 Gal(β-1,4)GlcNAc(β-1,4) GlcNAc(β-1,2) Man(α-1,3) GlcNAc(β-1,4) Man(α-1,3) Man(α-1,6) Man(α-1,6) Man(β-1,4)GlcNAc(β-1-4)GlcNAc(β-1,N)Asn

Man, mannose; GlcNAc, *N*-acetylglucosamine; NeuAc, neuraminic acid; Gal, galactose.

NeuAc located at the terminal position of oligosaccharide side chains are *N*-acetylated (NeuAcNAc) and are often referred to as sialic acid. Sialic acid is in fact the generic term given to a family of acetylated derivatives of neuraminic acid.

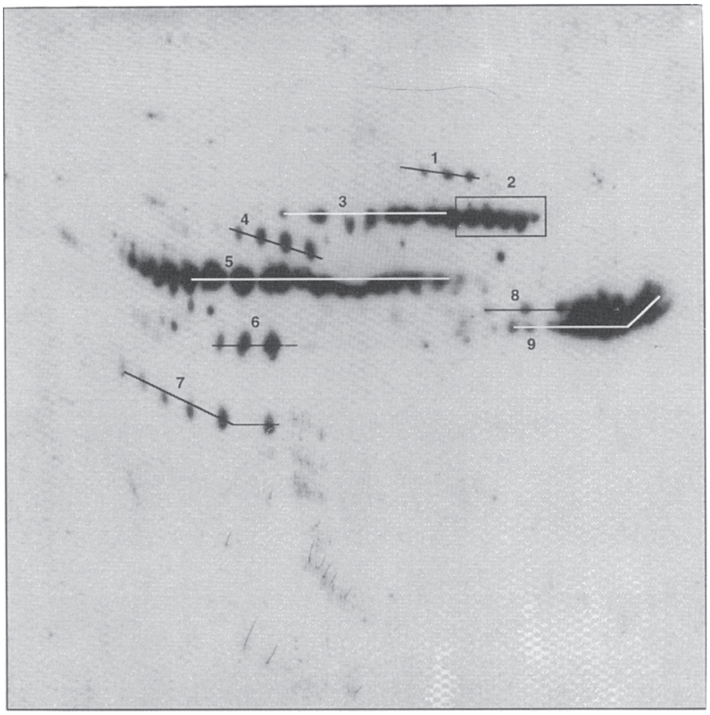


Fig. 3. Glycoprotein blot pattern of 2-D PAGE separation of plasma proteins (120 μg) probed with AAA (specific for fucose) and revealed using chemiluminescence reaction (5 s film exposure). 1) complement 3 α-chain, 2) transferrin, 3) IgM μ-chain, 4) hemopexin, 5) IgA α-chain, 6) fibrinogen γ-chain, 7) haptoglobin β-chain, 8) fibrinogen β-chain, 9) IgG γ-chain.

in **Fig. 5**. The presence of this common core structure reflects the fact that all these asparagine-linked oligosaccharides originate from the same precursor.

O-glycans are found frequently in mucins, but rarely in plasma glycoproteins. **Table 2** illustrates the 3 different groups of O-glycosyl protein glycans.

1.2. Lectins as a Tool for Glycoprotein Detection

Lectins are a class of carbohydrate-binding proteins, commonly detected by their ability to precipitate glycoconjugates or to agglutinate cells (some lectins react selectively with erythrocytes of different blood types). Lectins are present in plants, animals and microorganisms ***(14)***.

Each lectin binds specifically and non-covalently to a certain sugar sequence in oligosaccharides and glycoconjugates. Lectins are traditionally classified into specificity groups (mannose, galactose, N-acetylglucosamine, N-acetylgalactos-

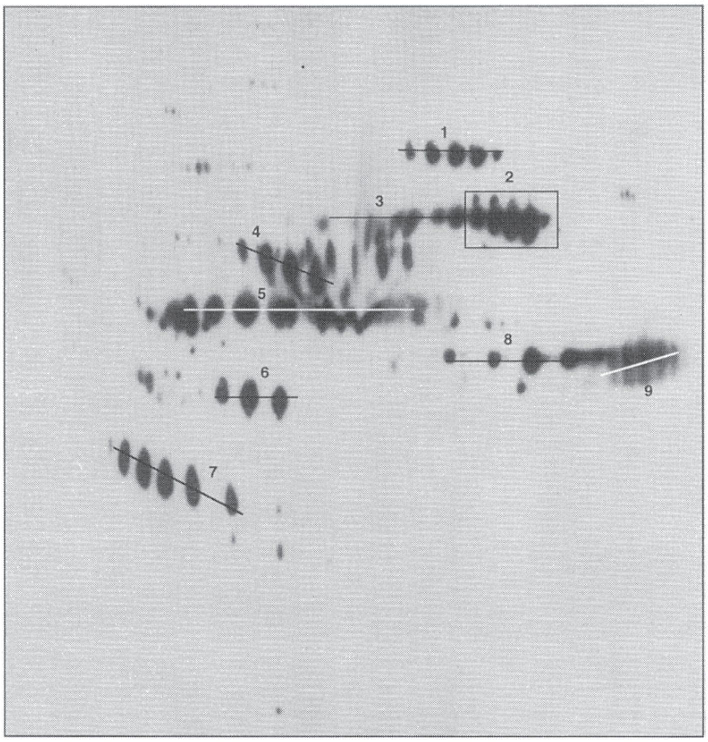
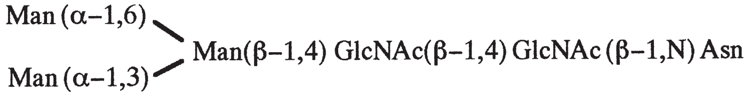


Fig. 4. Glycoprotein blot pattern of 2-D PAGE separation of plasma proteins (120 µg) probed with SNA (specific for neuraminic acid linked α-2,6 to galactose) and revealed using chemiluminescence reaction (30 s film exposure). 1) complement 3 α-chain, 2) transferrin, 3) IgM µ-chain, 4) hemopexin, 5) IgA α-chain, 6) fibrinogen γ-chain, 7) haptoglobin β-chain, 8) fibrinogen β-chain, 9) IgG γ-chain.

Table 2
Glycans *O*-Glycosidically Conjugated to Proteins

Type of *O*-glycosidic linkage	Examples of structures
Mucin type	NeuAc(α-2,6)GalNAc(α-1,3) Ser (or) Thr Gal(β-1,3)GalNAc(α-1,3) Ser (or) Thr The presence of such *O*-glycosidically linked moieties has been established for a number of plasma proteins (IgA, IgD, hemopexin, plasminogen, apoE)
Proteoglycan type	[GalNAc(β-1,4)GlcUA(β-1,3)]*n*GalNAc(β-1,4)GlcUA(β-1,3) Gal(β-1,3)Gal(β-1,4) Xyl(β-1,3)Ser They are generally linear polymers made up from repeating disaccharide units
Collagen type	Glc(β-1,2)Gal(β-1,5)OH-Lys

NeuAc, neuraminic acid; GalNAc, *N*-acetylgalactosamine; Gal, galactose; GlcUA, Glucuronic acid; Glc, glucose; Xyl, xylose.



$$\left.\begin{array}{r}\text{Man}\,(\alpha\text{-}1,6)\\ \text{Man}\,(\alpha\text{-}1,3)\end{array}\right\rangle \text{Man}(\beta\text{-}1,4)\ \text{GlcNAc}(\beta\text{-}1,4)\ \text{GlcNAc}\,(\beta\text{-}1,\text{N})\ \text{Asn}$$

Fig. 5. Oligosaccharide inner core common to all N-glycosylproteins.

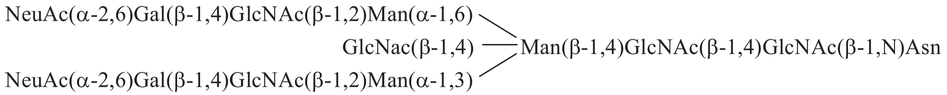
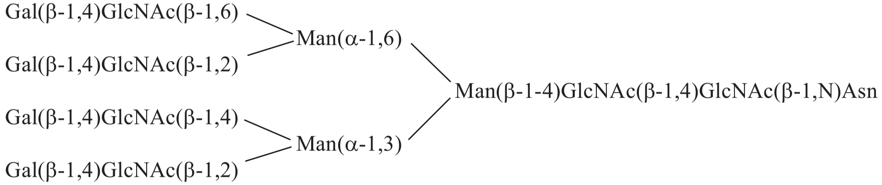
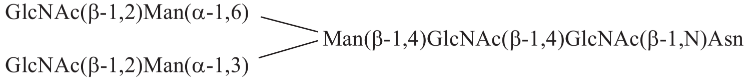
amine, fucose, neuraminic acid) according to the monosaccharide which is the most effective inhibitor of the agglutination of erythrocytes or precipitation of polysaccharides or glycoproteins by the lectin (fixation-site saturation method). Another method to determine the carbohydrate specificity of a given lectin consists in the determination of the association constant, by equilibrium analysis.

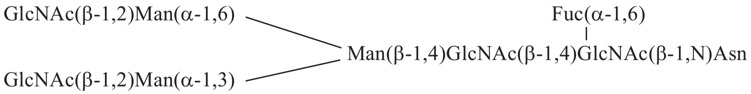
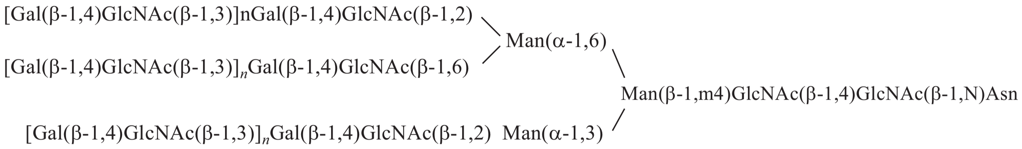
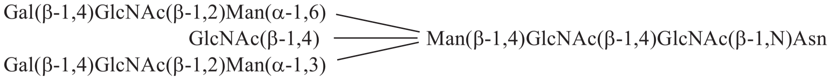
In most cases, lectins bind more strongly to oligosaccharides (di, tri and tetra saccharides) than to monosaccharides ***(15, 16)***. Therefore, the concept of "lectin monosaccharide specificity" should advantageously be replaced by that of "lectin oligosaccharide specificity." **Table 3** summarizes the carbohydrate specificity of lectins commonly used in biochemical/biological research, both in terms of the best monosaccharide inhibitor of the precipitation of polysaccharides or glycoproteins and in terms of the structure of oligosaccharides recognized by immobilized lectins. The interactions of lectins with glycans are complex and not fully understood. Many lectins recognize terminal nonreducing saccharides, while others also recognize internal sugar sequences. Moreover, lectins within each group may differ markedly in their affinity for the monosaccharides or their derivatives. They do not have an absolute specificity and therefore can bind with different affinities to a number of similar carbohydrate groups. Because lectin binding can also be affected by structural changes unrelated to the primary binding site, the results obtained with lectin-based methods must be interpreted with caution ***(2)***.

Despite these limitations, lectin probes do provide some information as to the nature and composition of oligosaccharide substituents on glycoproteins. Their use together with blotting technique provides a convenient method of screening complex protein samples for abnormalities in the glycosylation of the component proteins. Lectin blotting requires low amounts of proteins, is easy to perform and therefore is particularly indicated to analyze biological samples.

When the lectin-blotting method described hereafter is combined with the high resolution and reproducibility of 2-D PAGE and with the sensitivity of enhanced chemiluminescence, it is possible to identify rapidly the glycoproteins of interest by comparison with a reference 2-D PAGE protein map and to obtain reliable and reproducible results ***(30-32)***.

Table 3
Specificity of Lectins for Glycan Linked to Asparagine (*N*-Linked Oligosaccharide Chains)

Lectin	Monosaccharide specificity	Oligosaccharide specificity[a]
Wheat germ (WGA)	GlcNAc NeuAc	 NeuAc(α-2,6)Gal(β-1,4)GlcNAc(β-1,2)Man(α-1,6) GlcNac(β-1,4) Man(β-1,4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn NeuAc(α-2,6)Gal(β-1,4)GlcNAc(β-1,2)Man(α-1,3) Note: The GlcNAc (β-1,4) Man (β-1,4) GlcNAc (β-1,4) GlcNAc (β-1,N) Asn structure is important for tight binding of glycopeptides to a WGA agarose column ***(17)***. Neuraminic acids (NeuAc) are implicated as important factors in WGA interactions but the inhibitory effect of NeuAc is weaker than that of GlcNAc. The presence of clustering sialyl residues may be necessary for the stronginteraction of sialoglycoconjugates with WGA ***(18)***.
Ricinus communis (RCA1, RCA120)	Gal	 Gal(β-1,4)GlcNAc(β-1,6) Man(α-1,6) Gal(β-1,4)GlcNAc(β-1,2) Man(β-1-4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn Gal(β-1,4)GlcNAc(β-1,4) Man(α-1,3) Gal(β-1,4)GlcNAc(β-1,2) Note: RCA is specific for terminal β-galactosyl residues ***(19)***. It binds primarily to the terminal Gal (β -1,4) GlcNAc sugar sequence and much more weakly to the Gal (β-1,3) GalNAc sugar sequence ***(20)***. As galactose is the subterminal sugar in fully formed *N*-linked oligosaccharides, RCA provides a means of identifying the asialo forms of glycoproteins.
Concanavalin A (Con A)	Man	 GlcNAc(β-1,2)Man(α-1,6) Man(β-1,4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn GlcNAc(β-1,2)Man(α-1,3) Note: Con A interacts with glycoconjugates that have at least two nonsubstituted or 2-*O*-substituted α-mannosyl residues. It detects the core portion of *N*-linked oligosaccharide chains. The most potent hapten is illustrated here ***(17)***.

Lens culinaris (LCA)	Man	GlcNAc(β-1,2)Man(α-1,6) GlcNAc(β-1,2)Man(α-1,3) Fuc(α-1,6) Man(β-1,4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn Note: LCA is specific of mannose (α-1,3) or (α-1,6). Fucose (α-1,6) linked to GlcNAc is required for the reaction ***(3)***.
Galanthus nivalis (GNA)	Terminal Man	Man(α-1,2)(α-1,3) or (α-1,6)Man Note: GNA is particularly useful for identifying high mannose or hybrid type oligosaccharide structure because it does not react (unlike ConA) with biantennary complex type chains ***(21,22)***.
Datura stramonium (DSA)	GlcNAc	[Gal(β-1,4)GlcNAc(β-1,3)]nGal(β-1,4)GlcNAc(β-1,2) [Gal(β-1,4)GlcNAc(β-1,3)]$_n$Gal(β-1,4)GlcNAc(β-1,6) Man(α-1,6) Man(β-1,m4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn [Gal(β-1,4)GlcNAc(β-1,3)]$_n$Gal(β-1,4)GlcNAc(β-1,2) Man(α-1,3) Note: Datura lectin is specific for Gal-GlcNAc termini of complex oligosaccharides ***(22)***.
Peanut agglutinin (PNA)	Gal	Gal(β-1,3)GalNAc **refs.** ***22*** and ***23***
Phaseolus vulgaris (E-PHA) erythroagglutinating phytohemagglutinin	GlcNAc	Gal(β-1,4)GlcNAc(β-1,2)Man(α-1,6) GlcNAc(β-1,4) Gal(β-1,4)GlcNAc(β-1,2)Man(α-1,3) Man(β-1,4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn Note: A bisecting GlcNAc residue that links (β-1,4) to the β-linked mannose residue in the core is an essential and specific determinant for high-affinity binding to E-PHA-agarose column. Without this bisecting GlcNAc residue, a complex-type glycopeptide is not retained by E-PHA-agarose ***(24)***.

(continued)

Table 3
Specificity of Lectins for Glycan Linked to Asparagine (*N*-Linked Oligosaccharide Chains) *(continued)*

Lectin	Monosaccharide specificity	Oligosaccharide specificity[a]
Phaseolus vulgaris (L-PHA) leukoagglutinating phytohemagglutinin	GlcNAc	Gal(β-1,4)GlcNAc(B-1,2) Gal(β-1,4)GlcNAc(β-1,6) ⟩ Man (α-1,6) Man(β-1,4)GlcNAc(β-1,4)GlcNAc(β-1,N)Asn Gal(β-1,4)GlcNAc(β-1,4) Gal(β-1,4)GlcNAc(β-1,2) ⟩ Man(α-1,3)
		Note: The Gal (β-1,4) GlcNAc Man sugar sequence is essential for the binding of L-PHA (and E-PHA), as the interaction is completely abolished by the removal of the β-galactosyl residue in the peripheral portion ***(24)***.
Ulex europeus 1 (UEA1)	Fuc	Fuc(α-1,6) | GlcNAc(β-1,N)Asn **ref. *3***
Lotus tetragonolobus (LTA)	Fuc	Fuc(α-1,6) | GlcNAc(β-1,N)Asn Fuc(α-1,3) | Gal(β-1,4)GlcNAc Fuc(α-1,2) | Gal(β-1,4)GlcNAc **refs. *3*** and ***25***
Aleuria aurantia (AAA)	Fuc	Fuc(α-1,6) | GlcNAc(β-1,N)Asn Fuc(α-1,2) | Gal(β-1,4)GlcNAc Fuc(α-1,3) | Gal(β-1,4)GlcNAc
		Note: The AAA lectin is particularly useful for identifying fucose bound (α-1,6) to GlcNAc. Fucose bound (α-1,6) to GlcNAc in the core region is necessary for strong binding of AAA. Fucose (α-1,3) linked to GlcNAc and fucose (α-1,2) linked to Gal (β-1,4) GlcNAc in the outer chain react only very weakly with AAA. However, fucose (α-1,3) or (α-1,2), in addition to a fucose-linked (α-1,6) to the proximal core GlcNAc enhances the binding ***(17,26)***.

Sambucus nigra (SNA)	NeuAc	NeuAc(α-2,6)Gal Note: SNA binds specifically to glycoconjugates containing the (α-2,6)-linked NeuAc while isomeric structures containing terminal NeuAc in (α-2,3)- linkage are very weakly bound ***(27)***.
Maackia amurensis (MAA)	NeuAc	NeuAc(α-2,3)Gal Note: Immobilized MAA interacts with high affinity with complex-type tri- and tetraantennary Asn-linked oligosaccharides containing outer NeuAc linked (α-2,3) to penultimate Gal residues. Glycopeptide containing NeuAc linked only (α-2,6) to Gal do not interact with MAA ***(28)***.
Limulus polyphemus (LPA)	NeuAc	Terminal NeuAc **ref. *29***

Man, mannose; GlcNAc, *N*-acetylglucosamine; NeuAc, neuraminic acid; Gal, galactose; GalNAc: *N*-acetylgalactosamine; Fuc, fucose.

*a*Examples of carbohydrate structures recognized by the lectins commonly used in biochemical/biological research are presented in this table.

2. Materials

1. The transfer buffer for protein blotting by semi-dry method (see Chapter 59) or by tank method (*see* Chapter 58) is the Towbin buffer diluted 1:2 with distilled water: 12.5 m*M* Tris, 96 m*M* glycine and 10% (v/v) methanol ***(33)***.
2. PVDF membranes (0.2 mm, 200 × 200 mm) are supplied by Bio-Rad, the filter papers (chromatography paper grade 3 mm CHr) by Whatman and the nitrocellulose membranes (0.45 mm) by Schleicher & Shuell.
3. Blocking solution: 0.5% (w/v) Tween 20 in Phosphate buffered saline (PBS) ***(34).*** All further incubations and washing steps are carried out in the same blocking solution.
4. Phosphate buffered saline (PBS): 137 m*M* NaCl, 27 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4. A 10 fold concentrated PBS solution is prepared, sterilized by autoclaving and stored at room temperature for many weeks (PBS 10 X: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4. Add H_2O to 1 liter).
5. Biotinylated lectins are obtained from Boehringer-Mannheim. Sigma supply horseradish peroxidase (HRP) labelled Extravidin (a modified form of affinity-purified avidin) and alkaline phosphatase (AP) labelled Extravidin.
6. Two different detection methods are described: the chemiluminescent detection of horseradish peroxidase (HRP) activity using the luminol reagent (ECL Kit, Amersham International) and the conventional colorimetric reaction of alkaline phosphatase (AP) revealed by nitroblue tetrazolium/ bromochloro-indolyl phosphate (NBT/BCIP).

    a) Stock solutions of NBT and BCIP (Fluka): solubilize 50 mg NBT in 1 ml of 70% (v/v) dimethylformamide and 50 mg BCIP disodium salt in 1 ml dimethylformamide. These two solutions are stable when stored in closed containers at room temperature.
    b) Alkaline phosphatase buffer: 100 m*M* NaCl, 5 m*M* $MgCl_2$ and 100 m*M* Tris-HCl, pH 9.5 is prepared just before the colorimetric detection.
7. For the chemiluminescent detection of HRP activity, X-ray films (X-OMAT S, 18 × 24 cm) and a cassette (X-OMATIC cassette, regular screens) are available from Kodak.
8. For the development of X-ray film, an automatic developer machine is used (Kodak RP X-OMAT Processor, Model M6B). Manual development can also be done using the developer for autoradiography films (reference number P-7042, Sigma) and the fixer (reference number P-7167, Sigma).

3. Method

The use of gloves is strongly recommended to prevent blot contamination.

1. After electrophoresis and protein blotting procedures, the membrane is first washed with distilled water (3 × 5 min) and then treated for 1 hour at room temperature and under gentle agitation with 100 ml blocking solution (PBS containing 0.5% (w/v) Tween 20 (*see* **Note 3**).
2. The blot is then incubated for 2 h in biotinylated lectin, at a concentration of 1 µg/ml in the blocking solution, under agitation, in a glass dish and at room temperature. Twenty-five ml of lectin solution is used for the incubation of a membrane of 16.5 cm × 21 cm (*see* **Note 4**).
3. A washing step is then performed for 1 h with 6 changes of 200 ml of PBS-Tween 20.

4. Extravidin-HRP or Extravidin-AP diluted 1:2000 in the blocking solution is added for 1 h at room temperature under agitation. The membrane is then washed for 1 h with 6 changes of PBS-Tween 20.
5. The colorimetric reaction with alkaline phosphatase is carried out under gentle agitation by incubating the blot in the following solution: 156 µl BCIP stock solution, 312 µl NBT stock solution in 50 ml alkaline phosphatase buffer. The colorimetric reaction is normally completed within 10 to 20 min. The blotted proteins are colored in blue.
6. The chemiluminescence detection of peroxidase activity is performed according to the manufacturer's instructions (Amersham). The enhanced chemiluminescent assay involves reacting peroxidase with a mixture of luminol, peroxide and an enhancer such as phenol.

Five ml of detection solution 1 are mixed with 5 ml of detection solution 2 (supplied with the ECL Kit). The washed blot is placed in a glass plate and the 10 ml of chemiluminescent reagents are added directly to the blot with a 10 ml pipette, in order to cover all the surface carrying the proteins. The blot is incubated for 1 min at room temperature without agitation.

The excess chemiluminescent solution is drained off by holding the blot vertically. The blot is then wrapped in plastic sheet (Saran Wrap™), without introducing air bubbles and exposed (protein side up) to X-ray film in a dark room, using red safelights. The exposure time of the film depends on the amount of target proteins on the blot (*see* **Note 5**).

The development of the X-ray films can be done with an automatic developer or manually with the following protocol:

- The developer and fixer solutions are prepared according to the manufacturer's instructions (dilution 1:4 with distilled water).
- In a dark room, the film is attached to the film hanger and immersed in the developer solution until the bands (or spots) appear (the maximum incubation time is 4 min). The film should not be agitated during development.
- The hanger is immersed into water and the film is rinsed for 30 s to 1 min.
- The hanger is then placed in the fixer solution for 4 to 6 min. Intermittent agitation should be used throughout the fixing procedure.
- The film is washed in clean running water for 5 to 30 min.
- Finally, the film is dried at room temperature. All previous incubations are also undertaken at room temperature.

4. Notes

1. Instead of nitrocellulose, PVDF membrane can be used for lectin blotting.
2. **Fig. 1A** shows the plasma glycoprotein signals detected with WGA and generated on a film after chemiluminescence detection. **Fig. 1B** shows an identical blot stained with NBT/BCIP. As already reported ***(32)***, the same pattern of glycoproteins or glycoprotein subunits are revealed by both methods but the chemiluminescent detection system shows higher sensitivity (about 10

fold) than NBT/BCIP staining. The spots in the former case are more intense and the detection with enhanced chemiluminescence is more reliable and easier to control than the colorimetric reaction.

Albumin which does not contain any carbohydrate moiety represents a negative protein control in all blots.

Lectin blotting of plasma proteins with RCA, with AAA and with SNA are presented in **Figs. 2, 3,** and **4** respectively.

3. Generally, bovine serum albumin (BSA) or nonfat dry milk is used to block membranes. For lectin blotting, we have tested BSA, which has produced a very high background, probably due to glycoprotein contamination in the commercial preparations of this protein. When low fat dried milk was used, no glycoprotein signals were obtained on the blot after the chemiluminescent reaction. This could be attributed to the presence of biotin, that competed with biotinylated lectin and avoided its binding to the sugar moieties of glycoproteins ***(35)***. The PBS-Tween 20 blocking solution gave a very low background both for colorimetric and chemiluminescence detections.
4. The blot should be immersed in a sufficient volume of solution to allow a good exchange of fluid over its entire surface. The use of plastic bags to incubate protein-blots is not recommended because it often leads to uneven background and may cause areas without signal.
5. Most of the proteins in plasma are glycosylated. Therefore, a very short exposure time of the blot to X-ray film is needed. In general, when 120 µg of plasma proteins are loaded onto 2-D PAGE, the blot is exposed for a period of 3 to 30 s. The difference in the exposure time depends on the lectin used. For example, WGA lectin detects most of the N-linked glycoproteins and an exposure of only 3 s is sufficient ***(32)***. In this case, a longer exposure time leads to a very high background with no additional spot signal.

   For an unknown sample, the blot can be exposed for 1 and 5 min as a first attempt. The enhanced chemiluminescence using luminol (ECL Kit, Amersham) leads to a "flash" of light due to the addition of enhancers. The light emission on membrane peaks at 5–10 min after the addition of substrate and lasts for 2–3 h ***(36)***. Therefore longer exposure times (10 min – 3 h) may allow the detection of weak signals.

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A Sensitive Method to Quantitatively Detect Total Protein on Membranes after Electrophoretic Transfer Using Avidin- or Streptavidin-Biotin

William J. LaRochelle

1. Introduction

Since the initial publication by Towbin and coworkers ***(1)*** on the preparation of sodium dodecyl sulfate-polyacrylamide gel replicas, commonly referred to as protein blots, the transfer of proteins from inaccessible gel matrices to readily accessible solid supports such as nitrocellulose or nylon membranes has become widely accepted ***(2-4)***.

The detection of electrophoretically transferred or blotted proteins has ranged from the selective identification of an individual protein of interest to the general staining of total protein ***(5)***. Specific proteins are detected with probes such as antibodies that are either directly radiolabeled or conjugated to an enzyme ***(6,7)***. Alternatively, bound and unlabeled antibodies are amplified by a secondary antibody or affinity probe similarly conjugated. The use of biotinylated antibodies followed by avidin or streptavidin enzyme conjugates has become well accepted ***(8).*** Indeed, the high affinity interaction between biotin and streptavidin has been further popularized in such diverse methods as generating cRNA (riboprobes) for *in situ* detection of low-quantity expressed mRNA ***(9)***, immunohistochemical analysis of proteins, and high-throughput screening of aptamer microarrays ***(10)***.

Total protein detection is usually based on chemical affinity staining methods such as Coomassie blue ***(11)***, amido black ***(12)***, India ink ***(13)***, oxidation/derivatization of carbohydrate moieties ***(14,15)***, silver enhanced copper detection ***(16)***, or ponceau S ***(17)***. Other approaches often require the chemical modification of the polypeptide with hapten followed by detection with anti-hapten antibody and labeled/conjugated secondary antibody, protein A, or protein G ***(18)***. Fluorescent

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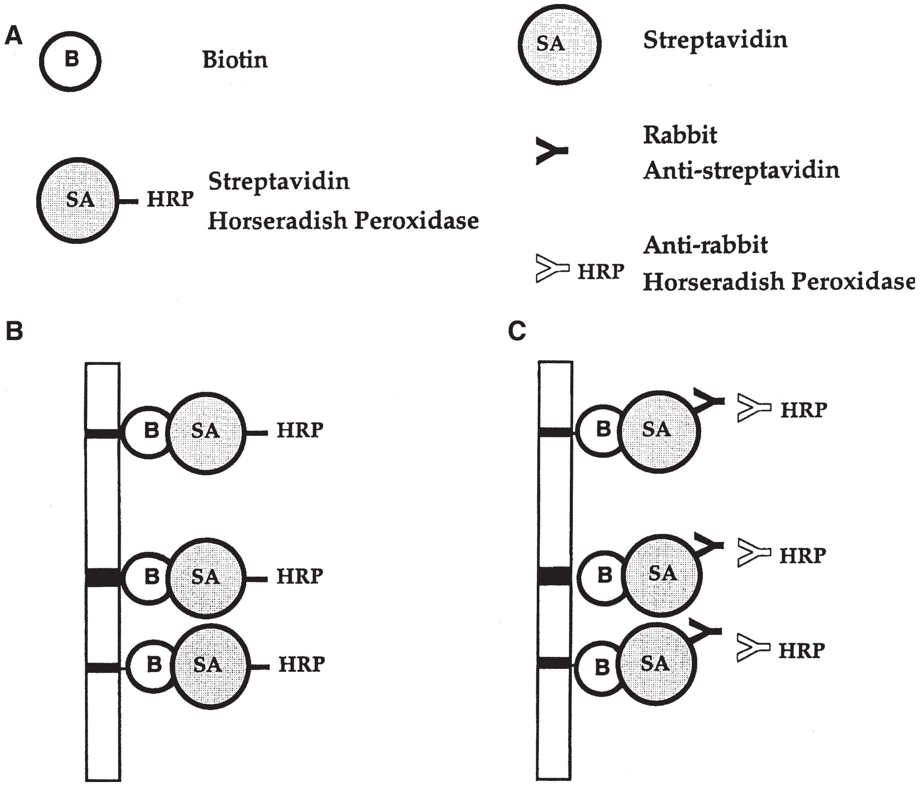
stains that improve on Sypro Ruby *(19)* such as LavaPurple or Deep Purple have been developed that improve the sensitivity for protein detection to as little as 1 ng per electrophoretically transferred band, but require fluorescent excitation capture by either a transilluminator or a fluorescent imager *(19,20)*.

Here, we exploit the high affinity and well characterized interaction of biotin with either avidin or streptavidin *(21-25)*. Briefly, proteins are resolved by SDS-PAGE, transferred to nitrocellulose membrane and the amino groups covalently derivatized *(23,26)* with sulfosuccinimidobiotin. Depending on the sensitivity


A
B
Biotin
SA
Streptavidin
SA
HRP
Streptavidin
Horseradish Peroxidase
Rabbit
Anti-streptavidin
HRP
Anti-rabbit
Horseradish Peroxidase
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SA
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HRP

Fig. 1. Schematic diagram of streptavidin or amplified anti-streptavidin staining of nitrocellulose replicas. Electrophoretically transferred proteins were biotinylated on a nitrocellulose replica depicted here as a strip blot (elongated rectangle). Panel A: Legend. Biotin, B; streptavidin, SA; horseradish peroxidase, HRP. Panel B: Schematic diagram of streptavidin staining method (**Subheading 3.1**). Total protein, shown as individual dark bands, was biotinylated and detected with streptavidin conjugated to horseradish peroxidase followed by the α-chloronaphthol color reaction. Panel C. Schematic diagram of amplified anti-streptavidin staining method (**Subheading 3.2**). After electrophoretic transfer and biotinylation, streptavidin binding to biotinylated proteins was detected with rabbit anti-streptavidin followed by goat anti-rabbit antibody conjugated to horseradish peroxidase as described in **Subheading 3.2**.

required, either of two techniques that are illustrated in **Fig. 1,** is used to stain the proteins that appear as dark bands against an essentially white background ***(26,27)***. The first method utilizes avidin or streptavidin conjugated to horseradish peroxidase and detects less than 25 ng of protein in a single band. The second technique, although slightly more lengthy, requires streptavidin amplification with anti-streptavidin antisera followed by a secondary antibody conjugated to horseradish peroxidase. The latter procedure detects less than 5 ng of protein per band.

The methods described here ***(26-30)*** permit the direct comparison of stained replicas with a duplicate blot that has been probed with for example, a specific antibody or ligand. The approach is rapid and possesses greater sensitivity than the commonly used dyes. The detection scheme is less costly and time consuming than the use of metal stains that in some instances possess greater sensitivity. Moreover, problems associated with gel shrinkage on drying or altered electrophoretic mobility of proteins caused by staining or derivatization prior to electrophoresis is avoided. Since our initial study (26), this approach has also proved useful in detection and labeling of DNA on membrane supports ***(31,32)***.

2. Materials

2.1. Avidin or Streptavidin Horseradish Peroxidase Staining

1. Distilled, deionized water.
2. Plastic trays rather than glass are preferred.
3. Nitrocellulose membranes, 0.45 μm (Bio-rad, Richmond, CA).
4. Sulfosuccinimidobiotin (Pierce Chemical Company, Rockford, IL) solution: 10 m*M* in 100 m*M* sodium bicarbonate, pH 8.0.
5. Avidin conjugated to horseradish peroxidase (Cappel Laboratories, Malvern, PA.
6. Streptavidin conjugated to horseradish peroxidase (Gibco-BRL, Gaithersburg, MD).
7. PBS: 10 m*M* sodium phosphate, 150 m*M* sodium chloride, pH 7.4.
8. Block solution: PBS containing 5% v/v newborn calf serum (Gibco-BRL, Gaithersburg, MD) and 3% w/v bovine serum albumin (BSA, Fraction V, 98–99%, Sigma Chemical Co., St. Louis, MO).
9. Wash solution: PBS containing 0.05% w/v Tween 80 (Sigma Chemical Co., St. Louis, MO).
10. PBS containing 0.05% w/v Tween 80 and 1.0% w/v BSA.
11. 1.0 *M* glycine-HCl pH 6.5.
12. α-chloronaphthol solution: PBS containing 0.6 mg/ml α-chloronaphthol (4-chloro-1-naphthol, Sigma) and 0.01% hydrogen peroxide.

2.2. Streptavidin/ Anti-Streptavidin Amplified Staining

1. Materials listed in **Subheading 2.1** excluding avidin or streptavidin-HRP.
2. Streptavidin (Gibco-BRL, Gaithersburg, MD).
3. Anti-streptavidin (Sigma Chemical Co., St. Louis, MO or Zymed Laboratories Inc., South San Francisco, CA).

4. Goat anti-rabbit immunoglobulin conjugated to horse radish peroxidase (Cappel Laboratories, Malvern, PA).

3. Method

3.1. Avidin or Streptavidin Horseradish Peroxidase Staining

1. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically transfer proteins to nitrocellulose membranes (*see* **Notes 1** and **2**). Rinse the nitrocellulose replicas three times and soak in 100 m*M* sodium bicarbonate pH 8.0 for 5 min (0.25 to 0.50 ml/cm^2 nitrocellulose). Typically, a 10 ml volume is used for a minigel replica. The solution volume should permit the filter to move freely upon agitation. Carry out all incubation and washing reactions using an orbital shaker or rocker platform at ambient temperature.
2. Transfer and submerge the replicas in the same volume of freshly prepared sulfosuccinimidobiotin solution for 45 min (*see* **Notes 3** and **4**).
3. Add 1 *M* glycine-HCl, pH 6.5 to a final concentration of 1 mM for approximately 5 min to quench the derivatization reaction.
4. Wash the filters 3 times (5 min each wash) with PBS to remove free sulfosuccinimidobiotin. Incubate filters 30 min with block solution.
5. Incubate the replicas for 1 h with either avidin conjugated to horseradish peroxidase (5 ug/ml) or streptavidin conjugated to horseradish peroxidase (1 ug/ml) diluted in PBS containing 1% BSA, 0.05% Tween 80 (*see* **Note 5**).
6. Wash filters 3 times for 15 min each time with the same volume of wash solution. Protein bands are visualized by immersing the replicas in α-chloronaphthol solution (*see* **Note 6**).
7. After allowing sufficient time for color development (usually 30 min), rinse the replicas with distilled water and dry between two sheets of dialysis membrane. The replicas may also be dried and stored in cellophane for future use.

3.2. Streptavidin/ Anti-Streptavidin Amplified Staining

1. If desired, an alternative procedure to amplify five-fold the detection method described above is utilized. First, biotinylate and block the nitrocellulose filters as described in **Subheading 3.1, Step 1** to **Step 4** of the avidin or streptavidin staining protocol.
2. Incubate blots with streptavidin (1 µg/ml) for 1 h in PBS containing 1% BSA, 0.05% Tween 80. Wash the replicas 3 times for 15 min each time with wash solution.
3. Next, dilute affinity purified rabbit anti-streptavidin IgG (0.5 µg/ml) in PBS containing 1% BSA, 0.05% Tween 80. Add solution to replica for 4 h or overnight if convenient. Wash replicas 3 times for 15 min each time with wash solution.
4. Incubate replicas with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase diluted (4 µg/ml) in PBS containing 1% BSA, 0.05% Tween 80 for 4 h. Wash replicas 3 times for 15 min each time with wash solution.
5. Protein bands are visualized by immersing the replicas in α-chloronaphthol solution for approximately 30 min (*see* **Note 6**). The replicas are rinsed with distilled water and dried between two sheets of dialysis membrane.

4. Notes

1. We found that this method is more sensitive for nitrocellulose membranes than for proteins transferred to Biodyne membranes by approximately tenfold due to the higher background staining of Biodyne membranes.
2. Because of the sensitivity of the staining, care should be taken to avoid protein contamination of replicas with fingertips, and so on. Gloves or forceps should be used.
3. This method chemically modifies the free amino groups of proteins bound to filters. Amine containing compounds such as Tris or glycine buffers will compete for biotinylation with the sulfosuccinimidobiotin. Blot transfer buffers that contain free amino groups such as Tris or glycine may be used, but must be thoroughly removed by soaking and rinsing as indicated in **Subheading 3.1**.
4. The staining is highly dependent on the sulfosuccinimidobiotin concentration. Sulfosuccinimidobiotin concentrations of greater than 10 μM have resulted in a dramatic decrease of protein staining intensity ***(19)***. In some instances it may be necessary to empirically determine the optimal concentration of sulfosuccinimidobiotin to use for staining particular proteins.
5. Little or no differences were observed when avidin conjugated to horseradish peroxidase was substituted for streptavidin conjugated to horseradish peroxidase. However, for some applications streptavidin may present fewer problems due to a neutral isoelectric point and apparent lack of glycosylation.
6. Sodium azide will inhibit horseradish peroxidase and accordingly must be removed before addition of the α-chloronaphthol solution.
7. All reagent concentrations were determined empirically and were chosen to give maximum staining sensitivity. In principle, our procedure can be used in double-label experiments in which all proteins on the replica are biotinylated and the same blot is then probed with radiolabeled antibody or protein A.
8. Enhanced chemiluminscence has been shown to increase the sensitivity of horseradish peroxidase calorimetric or dye based assays 10-fold ***(33)***.

Acknowledgments

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Detection and Quantification of Proteins on Immunoblots using Enhanced Chemiluminescence

Jennifer J. Young

1. Introduction

The technique of immunoblotting (Western blotting), following separation of proteins by gel electrophoresis, is widely used in molecular and cell biology ***(1,2)*.** It enables the proteins under study to be fixed on a membrane that is much easier to handle and probe than a polyacrylamide or agarose gel. Proteins are then detected by incubation of the membrane with an appropriate primary antibody for the protein concerned, followed by incubation with a secondary antibody raised to recognise epitopes on the constant region of the primary antibody. This secondary antibody can be conjugated to a radio-label, a fluorophore or an enzyme ***(3)*.** In the latter case an appropriate substrate is then used to generate a product which can be measured.

Early workers used radiolabels, particularly ^{125}I, to detect proteins on immunoblots ***(1)*.** The sensitivity was good and the method gave a quantifiable permanent record in the form of an image on X-ray film. However long exposure times were needed and this, combined with the associated safety and disposal problems, has made the technique less popular. Fluorescently-labelled antibodies have also been employed using a fluorescent laser scanner for measurement ***(4)*.** This system overcomes the problems of dealing with radio-active materials and has the advantage that the signal is read directly from the bound antibody but has limits in terms of sensitivity ***(5)*.** Enzymes conjugated to secondary antibodies offer a detection system where many molecules of product are generated for each enzyme-linked molecule of antibody bound to protein. This achieves amplification of the signal, giving increased sensitivity, without the need for the special handling conditions associated with radioactive tracers.

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The first detection systems developed for enzyme-linked antibodies were colorimetric, using chromogenic substrates which generated a product that could be visualised. Whilst easy to use and effective, they have several drawbacks. There is no permanent record as colours fade over time, membranes are not easily re-probed and sensitivity is still limited. Enhanced chemiluminescence (ECL) overcomes many of these limitations and is now widely used as a detection system, in preference to colorimetric methods. ECL is based on an enzyme-substrate system in which the reduction of hydrogen peroxide in alkaline conditions, catalysed by horseradish peroxidase (HRP), is accompanied by the oxidation of the chemiluminescent compound luminol, with the concomitant production of light (*see* ***Fig. 1***). In the presence of phenolic enhancers, the intensity of the light is increased (or enhanced) a 1000-fold and the time of emission extended ***(6)***. The outcome is a strong signal which peaks within 10 min and then decays slowly, having a half-life of 1 h. There is an excellent signal-to-noise ratio and the output can be satisfactorily recorded on photographic film. Subsequent densitometric analysis of the images can be used to quantify the protein bands. A similar system with alkaline phosphatase (AP) as the enzyme has also been developed, although this can suffer from problems of high background due to bacterial alkaline phosphatase contamination of buffers.

The use of enhanced chemiluminescence as a detection system offers a number of advantages:

- High sensitivity: using standard reagents 1 pg of protein can be detected, making it at least 10x more sensitive than colorimetric systems and 2–5x more sensitive than radioactive methods. Using some of the more recently developed reagents this sensitivity can be increased up to 200-fold (*see* **Note 11**).
- High resolution: the signal-to-noise ratio is low and a high contrast, easily quantifiable signal is generated.
- Speed: a strong light signal is generated rapidly which can be recorded within min.

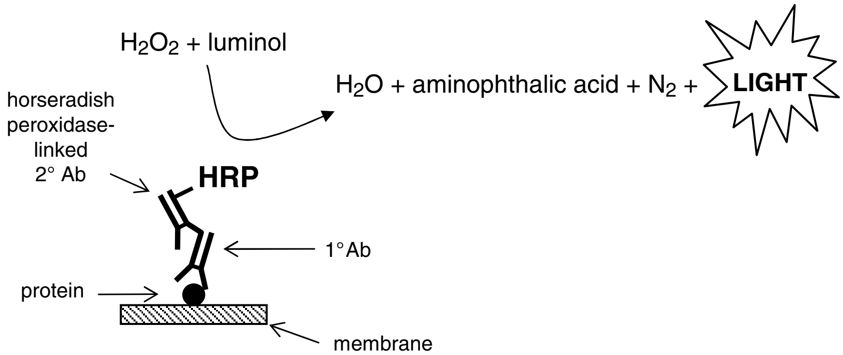
H_2O_2 + luminol
H_2O + aminophthalic acid + N_2 +
LIGHT
horseradish peroxidase-linked 2° Ab
HRP
1°Ab
protein
membrane

Fig. 1. Protein detection by chemiluminescence after immunoblotting.

- Permanent, quantifiable record: the film can be kept indefinitely and the amount of protein detected can be measured with a densitometer.
- Multiple images: the membrane can be repeatedly exposed to film, giving several copies of the image and an opportunity for optimisation.
- Conservative use of antibodies: the high sensitivity means a lower concentration of antibody is required.
- Versatility: agarose gels, in addition to one and two-dimensional polyacrylamide gels, can be probed.
- Nondestructive: the membrane can be reprobed many times with the same or different antibodies.

The technique of ECL is widely used as there are very many scenarios where proteins are studied both qualitatively and quantitatively. One example is the elucidation of signalling pathways within a cell under specific conditions when it is useful to monitor the induction, expression or phosphorylation of particular enzymes. Changes of proteins in different disease states can also be monitored. The example shown here is an immunoblot from an experiment that explored the possible contribution of lipopolysaccharide to the induction of the pro-inflammatory enzyme cyclo-oxygenase 2 at the site of a chronic wound (*see* **Fig. 2**).

2. Materials

2.1. Equipment

1. Electrophoresis equipment
2. Electroblotting equipment
3. Power pack
4. Forceps
5. Transfer membrane: for example nitrocellulose (Amersham Hybond™ ECL, GE Healthcare; WestClear™, GenScript; Immobilon P, Millipore), polyvinylidine difluoride (PVDF), (Amersham Hybond™ PAGE Healthcare)
6. Plastic cassette
7. SaranWrap™
8. X-ray film cassette
9. Autoradiography film: Amersham Hyperfilm™ ECL
10. Timer
11. Polaroid camera (Amersham, GE Healthcare) optional (*see* **Note 9**)
12. Software for densitometric analysis: Syngene GeneTools or Amersham ImageQuant™

2.2. Reagents

1. Acrylamide gels of the appropriate density prepared according to the method of Laemmli *(7)*.
2. Running buffer: 12 g Tris (0.05 *M*), 57.6 g glycine (0.384 *M*), 2.0 g SDS (0.1%) made up to 2 L in distilled water. No pH adjustment is necessary. It can be made up at x5 concentration and stored at room temperature for up to 3 months.

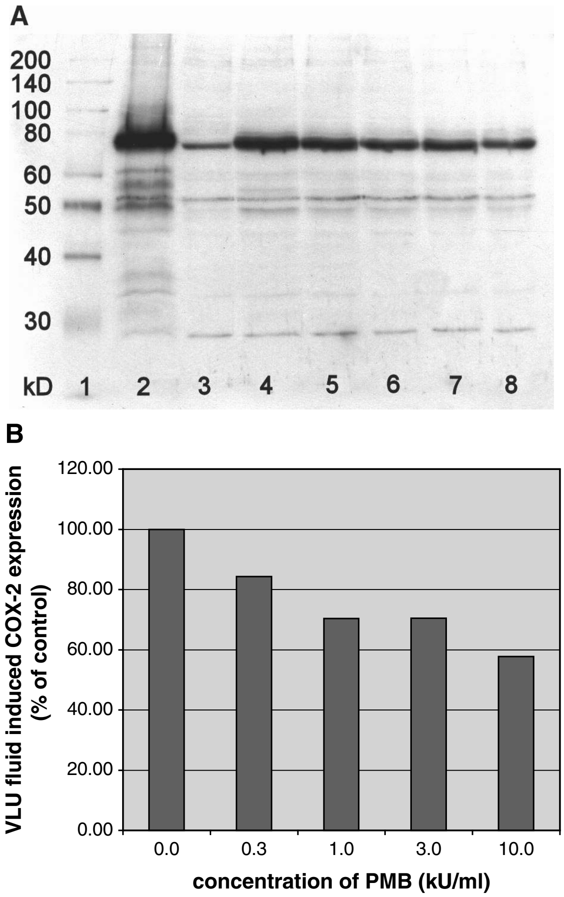

Fig. 2. Immunoblot and associated densitometry for the expression of cyclo-oxygenase 2 (COX-2) in J774 mouse macrophages treated for 18 hrs with venous leg ulcer (VLU) fluid. The VLU fluid had been pre-incubated with polymyxin B (PMB, which binds lipopolysaccharide), at a range of concentrations, for 1 hr prior to treatment of the cells. Depicted on the left of the immunoblot are the relative molecular masses of the standard proteins in the molecular weight marker (Biotinylated protein ladder detection pack, NE Biolabs). Lane 1 is the positive control for COX-2, lane 2 is lysate from untreated cells, lanes 4–8 are lysates from cells treated with VLU fluid which had been incubated with 0.0, 0.3, 1.0, 3.0, and 10.0 kU/ml of polymyxin B respectively. The graph shows the expression of enzyme as a percentage of that induced in cells dosed with untreated VLU fluid, The immunoblot was quantified using a document scanner and Syngene GeneTools software.

3. Sample buffer (*See* **Note 14**): 1 ml of 0.6M Tris-HCl, pH 6.8, 2 ml of 10% SDS solution, 1 ml glycerol, 0.1 ml of 2% bromophenol blue, can be stored at 2–8°C for 1 month. To use add the reducing agent 1*M* dithiothreitol (DTT) (0.1 ml DTT to 0.4 ml

stock). 1*M* DTT can be kept in one-use aliquots at −20°C. 2-mercaptoethanol can also be used as a reducing agent.

4. Samples
5. Positive control for the primary antibody (*see* **Note 1**).
6. Molecular weight marker (*see* **Note 2**).
7. Transfer buffer: Make up 15.14 g Tris (25 m*M*), 72.07 g glycine (192 m*M*) to 4L in distilled water and add 1L of methanol.
8. PBS-Tween: phosphate buffered saline with 0.05% Tween 20.
9. Blocking agent: for example 5% w/v nonfat dried milk, 1% w/v bovine serum albumin in PBS-Tween.
10. Primary antibody made up in PBS-Tween.
11. Secondary antibody conjugated to HRP or AP made up in PBS-Tween.
12. ECL reagents: Amersham ECL™ (GE Healthcare), Pierce ECL Western Blotting Substrate (Pierce), Immobilon™ Western HRP Substrate (Millipore), LumiSensor™ Chemiluminescent HRP Substrate Kit (GenScript) (*see* **Notes 10** and **11**)
13. Developer and fixer (Kodak GBX) diluted according to the manufacturer's instructions and stored in light-proof bottles. The prepared solutions can be kept at room temperature for at least 1 month provided the developer is not exposed to light other than the red light in the dark room.

3. Methods

3.1. Electrophoresis and Immunoblotting

The following is a brief summary of the protocols. These techniques are described in more detail in Chapters 58 and 59. The membrane should be handled using forceps and gloves at all times to avoid contamination.

1. Assemble the gel cassette and add running buffer.
2. Add the samples to an equal volume of sample buffer, heat to 95°C for 5 mins, centrifuge (10,000 g, 5 mins) and load onto the gel. Also load a sample of molecular weight marker and positive control for the primary antibody.
3. After separation by electrophoresis, transfer the proteins to a membrane (either nitrocellulose or PVDF) by immunoblotting. It is useful to cut one corner of the membrane before transfer to indicate the orientation of the blot. (*See* **Notes 6** and **12)**
4. The membrane can be probed immediately or air-dried and stored in a desiccator at 2–8°C for up to 3 months.

3.2. Probing the Membrane

The following is a recommended protocol but the solutions, times and temperatures can be varied to optimise conditions for the user's experimental system. All steps should be carried out on an orbital shaker.

1. Transfer the membrane to a plastic cassette containing blocking solution, with the surface that was touching the gel facing upwards. Incubate for 2 hrs at room temperature or overnight at 2–8°C (*see* **Note 4**)

2. Replace the blocking solution with primary antibody at the appropriate concentration and incubate for 2 hrs at room temperature or overnight at 2–8°C (*see* **Notes 4** and **13**)
3. Remove the primary antibody solution (*see* **Note 15**) and quickly rinse the membrane several times in PBS-Tween. Then wash the membrane more thoroughly by incubating 4x in PBS-Tween, placing the cassette on the shaker for 10 mins for each wash (*see* **Note 4**)
4. Add secondary antibody solution at the appropriate concentration and incubate at room temperature for 30 mins to 1 h. If using a molecular weight marker that requires a detection antibody add it to the solution at this stage (*see* **Notes 2** and **4**)
5. Remove the secondary antibody solution and wash the membrane in PBS-Tween, as described in step 4.

3.3. Detection by Enhanced Chemiluminescence

1. Remove excess buffer from the membrane by holding it vertically with forceps and touching the edge against a piece of paper towel. Further drying can be achieved by placing the membrane in a clean plastic cassette (protein side upwards) and then lifting it repeatedly, each time wiping the cassette beneath the membrane with a paper towel. Leave the membrane in the cassette (*see* **Note 4**)
2. Prepare the substrate according to the manufacturer's instructions. 1 ml of substrate will be sufficient for each membrane if it was cut to match the size of a minigel. Larger membranes will require more and the volume can be increased accordingly.
3. Using a Gilson pipette spread the substrate over the surface of the membrane, tilting the cassette gently to ensure it is evenly covered (*see* **Note 6**). Incubate for 1 min.
4. Using forceps dry the membrane by holding it vertically and touching the edge against a piece of paper towel. Further drying can be achieved as described above.
5. Place the membrane on a piece of SaranWrap™ and carefully fold over the wrap to cover the membrane, making sure there are no creases.
6. Place the membrane in the X-ray film cassette, protein side upwards. It can be taped in place if necessary.
7. Cut a piece of film slightly larger than the membrane and place it on top.
8. Close the cassette and expose the membrane for 5 min. This is a useful time for a first exposure but the time can be adjusted for subsequent images according to the result (*see* **Note 3**)
9. Remove the film using forceps and gently agitate in developer solution, watching for the appearance of bands (*see* **Notes 4** and **5**)
10. As soon as the bands appear transfer to a tray of water and wash off the developer by agitating gently.
11. Transfer to fixer and leave to fix. The background to the film will become clear when this stage is completed.
12. Thoroughly wash off fixer by holding the film under a running tap and leave to dry.
13. Further exposures can be carried out according to the results (*see* **Note 3**).
14. It is recommended that all images are labelled immediately with samples, date and antibody used.
15. If appropriate the membrane can be re-probed (*see* **Note 16**).

3.4. Densitometric Analysis of Immunoblot

The photographic film can be analysed semiquantitatively in one of several ways. Either the image can be captured on a gel documenter (SynGene Gene Genius) using SynGene software and then analysed using SynGene GeneTools. Alternatively the image can be scanned using a document scanner and then analysed by GeneTools software. Densitometric analysis is also possible in Photoshop but is more tedious done this way and is only useful for occasional images. Semi-quantitative analysis is useful when a comparison to a control is required, for instance when looking at the induction of an enzyme within a cell or for tracking the phosphorylation of a particular protein on a signalling pathway.

Several films exhibit a linear response to the light produced by ECL. It is therefore possible for a more rigorous quantification to be achieved. In addition the range can be extended to increase accuracy at the lower levels by pre-flashing the film prior to exposure (*see* **Note 17**). A range of standards containing known amounts of the protein under investigation should be loaded onto the gel, in addition to at least two dilutions of the unknown samples. The time of exposure to the film should then be optimised so that the lowest concentration of standard protein is just visible. After scanning and densitometric analysis a graph of peak area against protein concentration can be plotted and the concentration of the unknown samples determined.

A charge coupled device (CCD) camera can be used as an alternative system for capturing the light output. These convert the light emitted from the blot to an electrical signal which is then converted to a digital signal to give a quantifiable value *(8)***.** In some cases they can provide better sensitivity than film and they have a wider dynamic range, giving increased options for quantification.

4. Notes

The methods of electrophoresis and immunoblotting are addressed in Chapters 58 and 59 and the associated notes should also be read.

For detection by ECL please note the following.

1. A positive control for the primary antibody should always be included on the gel. This provides a way of ensuring that if no bands are seen in the experimental samples it is not due to lack of antibody binding.
2. Several types of molecular weight markers can be used and everybody has their favourites. Common ones are pre-stained markers which can be seen on the membrane. These have the advantage of showing whether the transfer has been successful prior to exposure to antibody. The position of the standard proteins can be noted on the image by lining it up with the membrane after development and recording their position with a permanent marker. Other types of molecular weight marker are sold as kits which include a labelled

secondary antibody for the standard proteins. This secondary antibody can be added at the same time as the secondary antibody for the protein under study. These markers usually require at least 3 mins exposure to the photographic film and are not always appropriate if a high affinity primary antibody is being used or if an abundant protein is being studied. However the primary antibody solution can always be used at a lower concentration and/or lower sample volumes applied to the gel to get round this.

3. Exposure times vary according to the relative abundance of the protein and the affinity of the primary antibody. If no bands are seen with a relatively short exposure time it is always worth leaving the membrane exposed to film for 20–30 mins.
4. A high background on the area of the photographic film that corresponds to the membrane can be caused by a variety of things. Firstly it should be noted that polyclonal primary antibodies, by their very nature, are likely to cause higher levels of nonspecific binding than monoclonal antibodies. However high background can be reduced by:

   - More thorough washing. Do not be afraid to spray directly onto the membrane with a squeezy bottle containing the PBS-Tween when carrying out the washes.
   - Better blocking. If blocking was carried out at room temperature, try an overnight incubation. This is particularly useful if using a detergent like Tween 80 rather than a protein as the blocking agent. Alternatively try a different blocking solution. It should be noted that not all nonfat milk is the same. The author has found that "own-label" products sometimes contain a low level of fat which seems to make them less efficient at blocking the empty sites on the membrane.
   - Reducing the concentration of the antibodies, particularly the secondary one.
   - Reducing the development time.
   - Checking that the secondary antibody is not too old. They do degrade over time and high background is a consequence of this.
   - More thorough drying of the membrane prior to application of ECL reagent. The presence of Tween 20 can cause increased background.
   - By including either nonfat milk (1–5%) or BSA (1%) in the primary and secondary antibody solutions.

5. High background across the entire film is usually caused by old developer and is rectified by preparing a fresh solution.
6. Bubbles can be seen on the image for several reasons. Either air or buffer was trapped between the membrane and the gel during transfer, the ECL reagent was unevenly applied to the membrane or the membrane was allowed to partially dry out at some stage.

7. White bands on the film are indicative of excess protein or antibody, leading to rapid substrate depletion.
8. If the bands on the image are very diffuse it may be because the membrane was inadvertently turned over during the procedure and the substrate applied to the side which was not in contact with the gel.
9. For initial experiments a Polaroid™ camera (Amersham, GE Healthcare) can be used to obtain an image from blots generated from mini-gels. Initial exposure time should be 1–2 mins. It is quick and easy and the resulting image gives a good first indication of the results of the experiment and can show whether it is worth using the conventional method to get a better quality image for possible publication.
10. Whilst there is a range of reagents commercially available, ECL reagent can also be prepared in the laboratory. The components are: 10 n*M* luminol in 20 m*M* Tris-HCl, pH 8.5; 5 n*M* 4-iodine-phenol in 20 m*M* Tris-HCl, pH 8.5; 20 n*M* aqueous H_2O_2 solution.
11. More sensitive ECL reagents have now been developed by some suppliers and are useful for visualising proteins at very low levels or when the primary antibody is in short supply. Examples are Amersham ECL Plus and Amersham ECL Advance™ (GE Healthcare). There are also chemifluorescent reagents such as Amersham ECL Plus™ Chemifluorescent (GE Healthcare).
12. If no image at all is seen this could indicate that the power pack was incorrectly connected or the gel membrane sandwich put in the cassette the wrong way around. The efficiency of the transfer can be checked before antibody application by incubation of the membrane in Ponceau S dye solution for a few minutes followed by washing with distilled water. Whilst the proteins under study may be of too low an abundance to be picked up, the standard proteins in the molecular weight marker should still be seen. In addition the gel can be stained with Coomassie Brilliant Blue dye (*see* Chapter 21).
13. Alternatively, if no image was seen, check that a primary antibody suitable for immunoblotting was used. The proteins in this procedure are denatured and antibodies raised to epitopes on native proteins may not bind to them.
14. For samples prepared from cell lysates, if the antibody affinity is low or the protein content of the sample low, a modified method of preparing the samples can be used to increase the protein content. By using a lysis buffer made up from the sample buffer without the dye and reducing agent to lyse the cells, only the latter two components need to be added to the samples to prepare them for electrophoresis. This reduces the overall volume, maximising the protein content of the sample.
15. Primary antibodies can be stored and reused. However care must be taken with the use of sodium azide to inhibit bacterial growth as it interferes with horse-

radish peroxidase. It can be used successfully but the amount must be kept to an absolute minimum and thorough washing prior to secondary antibody incubation adhered to.

16. Membranes can be re-probed. Sometimes, if the second protein under investigation is of a very different molecular weight, the membrane can just be thoroughly washed in PBS-Tween and reincubated with the appropriate primary antibody. Alternatively the membrane can be completely stripped of primary and secondary antibodies by submerging it in stripping buffer (62.5 m*M* Tris-HCl pH 6.7, 100 m*M* 2-mercaptoethanol, 2% SDS) and incubating at 50°C for 30 mins with occasional agitation. The membrane should then be washed in PBS-Tween (2 × 10 mins) and blocked by incubation in blocking solution for 1 hr at room temperature. It is also possible to buy stripping solution in kits, for example ReBlot plus™ Western Blot Recycling Kit (Millipore). Membranes can be probed 5–10 times and should be stored wet, wrapped in SaranWrap™, at 2–8°C between each immunodetection.
17. Preflashing can be carried out using a modified flash unit that has been calibrated (by adjusting its distance from the film) to raise the optical density of the film by 0.1–0.2 OD units above that of the standard film. The flash duration should be about 1 msec.

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78

Reutilization of Western Blots After Chemiluminescent or Autoradiographic Detection

Scott H. Kaufmann

1. Introduction

Western blotting (also called immunoblotting) is a widely utilized laboratory procedure that involves formation and detection of antibody-antigen complexes between antibodies that are initially in solution and antigens that are immobilized on derivatized paper ***(1-3)*** This procedure is most commonly performed by sequentially subjecting a complex mixture of polypeptides to three manipulations: i) electrophoretic separation, usually through polyacrylamide gels in the presence of sodium dodecylsulfate (SDS); ii) electrophoretic transfer of the separated polypeptides to thin sheets of nitrocellulose or polyvinylidene fluoride (PVDF); and iii) reaction of the sheets sequentially with one or more antibody-containing solutions. Because these are laborious, low throughput procedures, there has been considerable interest over the past 15 years in increasing the information gained from immunoblots. Various approaches to this problem have included removal of bound antibodies so that immobilized polypeptides can be reprobed with additional antisera (last reviewed in ***(4)***), the sequential ***(5)*** or simultaneous ***(6)*** use of multiple antisera to detect different antigens, and the development of protein microarrays that can be used to simultaneously detect antibodies to a variety of immobilized antigens ***(7, 8)*** or quantitate binding of labeled polypeptides from complex mixtures to immobilized antibodies ***(9, 10)***. All of these approaches are variations on the basic immunoblotting procedure developed almost 30 years ago.

Because of their versatility, immunoblotting approaches are widely employed in biological studies. With a high quality antiserum or monoclonal antibody, this approach can determine whether an antigen of interest is present in a particular

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biological sample, monitor the purification of the antigen, or assess the location of epitopes within the antigen after chemical or enzymatic degradation. If suitable immunological probes are available, blotting can likewise be utilized to search for polypeptides that bear a particular physiological or pathological posttranslational modification, e.g., phosphorylation ***(11)*** or nitrosylation of tyrosine ***(12)***, covalently attached glycosylphosphatidylinositol ***(13)***, or modification by D-penicillamine metabolites ***(14)***. Conversely, immunoblotting can also be utilized to determine whether antibodies that recognize a particular antigen are present in a sample of biological fluid. For example, western blotting has become a method of choice for confirming that antibodies to human immunodeficiency virus-1 ***(15)***, Herpes simplex virus-2 ***(16)*** or *B. burgdorferi* ***(17)*** are present in patients suspected of harboring these pathogens. Likewise, immunoblotting or variations on this procedure are utilized to detect autoantibodies present in the cerebrospinal fluid of patients with multiple sclerosis ***(18, 19),*** characterize antigens recognized by autoantibodies in various dermatological and rheumatological disorders ***(7, 20, 21)***, and identify allergens that plague atopic individuals ***(22)***.

There are certain circumstances in which it is convenient to be able to probe immunoblots for the presence of multiple antigens. If immunoblotting suggests an unexpected subcellular distribution for a polypeptide, for example, reprobing the blot with a reagent that recognizes a second polypeptide is essential in order to confirm that the samples have been properly prepared, loaded, and transferred. Likewise, if the polypeptides being analyzed are derived from a precious source (e.g. pathological tissue, biological fluid, or organism that is not readily available), it is sometimes important to reutilize the blots.

1.1. Reutilization of Blots Without Removal of Antibodies

When two antigens of interest can be readily distinguished by size, blots can be probed with two different immunological reagents simultaneously. Bound primary antibody is then visualized by probing with one or more enzyme-coupled or radiolabeled secondary antibodies as needed. Interpretation of results obtained in this manner can be difficult, however, if one or more of the immunological reagents cross-reacts with multiple polypeptides on the blot or one of the polypeptides of interest exists as multiple species, e.g., splice variants or posttranslationally modified forms. A recently described alternative to this procedure involves the use of primary antisera raised in several different species followed species-specific secondary antibodies coupled to fluorophores with distinguishable spectral properties ***(6).*** This approach has the advantage of allowing unequivocal detection of the species that react with each primary antibody. Its disadvantages, however, include the need for primary antisera raised in different species and the need for specialized imaging equipment, which some centers do not have.

An alternative to simultaneous probing involves sequential probing of blots with different reagents. Earlier studies demonstrated that sodium azide inactivates horseradish peroxidase *(23, 24)*. Building on these observations, we demonstrated that blots can be sequentially reacted with multiple different peroxidase-coupled secondary antibodies without interference provided that the primary antibody solutions contain sufficient azide to inactive the enzyme bound to previously added secondary antibody *(25)*. This process is illustrated in **Fig. 1,** where the same piece of nitrocellulose was sequentially reacted with

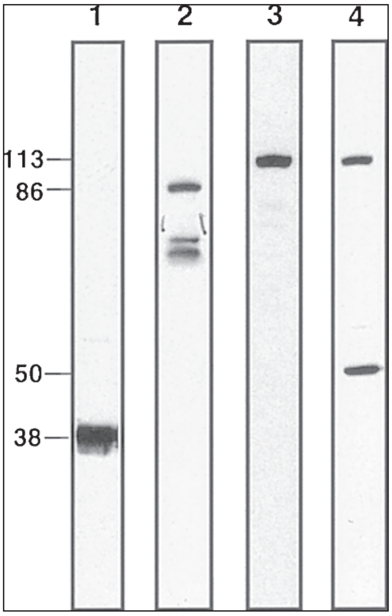

Fig. 1. Reutilization of immunoblots without intervening dissociation of antibodies. After staining with fast green FCF and coating with TSM (**Subheading 3.1, steps 8–11**), a single strip containing total cellular protein from 3 × 10^5 K562 human leukemia cells was sequentially subjected to immunoblotting using the following primary (secondary) reagents: chicken anti-B23 (peroxidase-coupled goat anti-chicken IgG), rabbit anti-protein kinase Cδ (peroxidase-coupled goat anti-rabbit IgG), mouse anti-poly(ADP-ribose) polymerase (peroxidase-coupled goat anti-mouse IgG), and mouse anti-procaspase-9 (peroxidase-coupled goat anti-mouse IgG). Bound secondary antibodies were detected using enhanced chemiluminescence reagents. At each step, the primary antibody solution contained 1 mM sodium azide. Note that the peroxidase bound to the blot at each step is not catalytically active at subsequent steps (cf. lanes 1–3), whereas the previously bound primary antibodies react with fresh secondary antibodies at subsequent steps (cf. lanes 3, 4). Numbers at left, molecular weights of the respective proteins in kDa.

immunological reagents raised in chickens, rabbits and mice. With successive use of reagents raised in different species, the signal obtained at each step is unaffected by the prior reactions (**Fig. 1,** lanes 1–3) even though the previously bound antibodies remain attached to the blot. It is important to stress, however, that each of these probings involves a different secondary antibody. When the same blot is subsequently probed with a second mouse IgG monoclonal antibody, the previously bound mouse IgG is detected by the peroxidase-coupled anti-mouse IgG (**Fig. 1,** lanes 3 and 4). Thus, sequential probing works well when multiple primary antibodies raised in different species are available or when antigens of different molecular weights can be unequivocally detected using antibodies from the same species. This approach can become problematic, however, if the antigens of interest migrate similarly or the immunological reagents cross-react with multiple species of different molecular weights. Under these circumstances, removal of antibodies from blots might be required between sequential probing.

1.2. Removal of Antibodies After Blotting

Methods for removing bound antibodies before probing with a new immunological reagent are patterned after methods for reutilizing Southern and Northern blots ***(26)***. In principle, the goal is to remove antibodies from the blot without removing the antigen of interest. The original methods developed with this purpose in mind (reviewed in **ref.** ***27***) involved the covalent binding of target polypeptides to derivatized paper so that subsequently bound antibodies could be solubilized under denaturing conditions without eluting the antigens of interest. Unfortunately, these techniques never gained widespread acceptance because of the fragility of derivatized paper.

Polypeptides are more commonly immobilized on nitrocellulose or PVDF, solid supports that are thought to bind macromolecules noncovalently. The critical issue then becomes the identification of conditions that solubilize antibodies without solubilizing the target polypeptides. Some reports have indicated that treatment of nitrocellulose blots with glycine (pH 2.2) at room temperature ***(28)*** or 8 M urea at 60°C ***(29)*** can remove antibodies and permit reutilization of blots. Other studies have indicated that these techniques disrupt low-affinity antibody-antigen interactions but not high-affinity interactions ***(28, 30)***

Subsequent observations allowed the development of a more widely applicable technique for the removal of antibodies from Western blots. In particular, treatment of nitrocellulose with acidic solutions of methanol was observed to “fix” transferred polypeptides to nitrocellulose ***(31, 32)***. Polypeptides treated in this fashion remain bound to nitrocellulose even during treatment with SDS at 70–100°C under reducing conditions ***(30, 31)***. These observations facilitated development of a simple procedure for removal of peroxidase-coupled antibodies after detection of antigens by enhanced chemiluminescence (*see* **Subheading 3.3**).

Because of the increased sensitivity of autoradiographic detection, merely solubilizing antibodies in SDS is sometimes not sufficient to permit reutilization of blots probed with radiolabeled antibodies. We previously observed that treatment of blots with a large excess of irrelevant protein immediately prior to drying and autoradiography facilitates subsequent removal of radiolabeled antibodies from nitrocellulose ***(30)***. Based on this observation, a stripping technique that allows the reutilization of Western blots after reaction with a wide variety of radiolabeled antibodies or lectins (**Subheadings 3.3** and **3.4**) was developed **(30)**.

2. Materials

1. Apparatus for transferring polypeptides from gel to solid support. (Design principles are reviewed in ***refs. 2*** and ***3***).
   a. Reservoir-type electrophoretic transfer apparatus (Hoefer Scientific, Holliston, MA) or equivalent.
   b. Polyblot semidry blotter (Amersham Biosciences) or equivalent.
2. Paper support for binding transferred polypeptides.
   a. Nitrocellulose.
   b. Nylon (e.g., Genescreen from New England Nuclear, Boston, MA, or Nytran from Schleicher and Schuell, Keene, NH).
   c. PVDF (e.g., Immobilon from Millipore, Bedford, MA).
3. Fast green FCF for staining polypeptides after transfer to solid support.
4. Penicillin 10,000 units/ml and streptomycin 10 mg/ml.
5. Reagents for electrophoresis (acrylamide, bisacrylamide, 2-mercaptoethanol, sodium dodecylsulfate) should be electrophoresis grade.
6. All other reagents (Tris, glycine, urea, methanol) are reagent grade.
7. Transfer buffer: 0.02% (w/v) sodium dodecylsulfate (SDS), 20% (v/v) methanol, 192 mM glycine, and 25 mM Tris base. Prepare enough buffer to fill the chamber of the transfer apparatus and a container for assembling cassette.
8. Fast green stain: 0.1% (w/v) Fast green FCF in 20% (v/v) methanol- 5% (v/v) acetic acid. This stain is reusable. Prepare 50–100 ml per blot.
9. Fast green destain: 20% (v/v) methanol in 5% (v/v) acetic acid.
10. TS buffer: 150 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4. This can be conveniently prepared as a 10X stock (1.5 *M* NaCl, 100 m*M* Tris-HCl, pH 7.4). The 10X stock can be stored indefinitely at 4°C and then used to prepare 1X TS buffer, TSM buffer, and the other buffers described below.
11. TSM buffer: TS buffer containing 10% (w/v) powdered milk, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 m*M* sodium azide. This buffer can be stored for several days at 4°C. Note that sodium azide is poisonous and can form explosive copper salts in drain pipes if not handled properly.
12. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, pH 7.4. This can be conveniently prepared as a 10-fold concentrated stock at pH 7.0 and stored at 4°C. Upon dilution the pH will be 7.4.
13. PBS containing 2 *M* urea and 0.05% (w/v) Nonidet P-40. Prepare 300 ml per blot by combining 0.15 gm of Nonidet P-40, 30 ml of 10X PBS buffer, 75 ml of 8 *M*

urea (freshly deionized over BioRad AG1X-8 mixed bed resin to remove traces of cyanate) and 195 ml of water.

14. PBS buffer containing 0.05% (w/v) Nonidet P-40. Prepare 300 ml per blot.
15. PBS buffer containing 3% (w/v) nonfat powdered milk. Prepare 25 ml per blot.
16. Blot erasure buffer: 2% (w/v) SDS, 62.5 m*M* Tris-HCl (pH 6.8 at 21°C), and 100 m*M* 2-mercaptoethanol. The SDS/Tris-HCl solution is stable indefinitely at 4°C. Immediately prior to use, 2-mercaptoethanol is added to a final concentration of 6 µl/ml.
17. Primary antibody.
18. Reagents for detection by chemiluminescence. This approach requires enzyme-coupled secondary antibody and a substrate that becomes chemiluminescent as a consequence of enzymatic modification, e.g., peroxidase-coupled secondary antibody and luminol (Supersignal® (Pierce, Rockford, IL) enhanced chemiluminescence kit or equivalent) (*see* Chapter 78).
19. In lieu of enzyme-coupled secondary antibody, (^{125}I)-labeled secondary antibody can be used. Secondary antibodies can be labeled as previously described (30) or purchased commercially. Radiolabeled antibodies should only be used by personnel trained to properly handle radioisotopes.

3. Methods

3.1. Transfer of Polypeptides to Nitrocellulose

The following description is appropriate for transfer in a transfer reservoir. If a semidry transfer apparatus is to be used, follow the manufacturer's instructions (*see* **Note 1**).

1. Perform SDS-polyacrylamide gel electrophoresis using standard techniques (*see **ref. 33*** for description of this method).
2. Wear disposable gloves while handling the gel and nitrocellulose at all steps. This avoids cytokeratin-containing fingerprints.
3. Cut nitrocellulose sheets to a size slightly larger than the polyacrylamide gel (*see* **Note 2**).
4. Fill the transfer apparatus with transfer buffer (*see* **Note 3**).
5. Fill a container large enough to accommodate the transfer cassettes with transfer buffer. Assemble the cassette under the buffer in the following order:
   a. Back of the cassette.
   b. Two layers of filter paper.
   c. The gel.
   d. One piece of nitrocellulose– gently work bubbles out from between the nitrocellulose and gel by rubbing a gloved finger or glass stirring rod over the surface of the nitrocellulose.
   e. Two layers of filter paper– again, gently remove bubbles.
   f. Sponge or flexible absorbent pad.
   g. Front of the cassette.

6. Place the cassette in the transfer apparatus so that the front is oriented toward the POSITIVE pole.
7. Transfer at 4°C in a cold room with the transfer apparatus partially immersed in an ice-water bath. Power settings: 90 V for 5–6 h or 60 V overnight.
8. Place the fast green stain in a container with a surface area slightly larger than one piece of nitrocellulose (*see* **Notes 4** and **5**). After the transfer is complete, place all the pieces of nitrocellulose in the stain and incubate for 2–3 min with gentle agitation. Decant the stain solution, which can be reused.
9. Destain the nitrocellulose by rinsing it for 3–5 min in fast green destain solution with gentle agitation. Decant the destain, which can also be reused. Rinse the nitrocellulose four times (1–2 min each) with TS buffer (200 ml/rinse).
10. Mark the locations of lanes, standards, and any other identifying features by writing on the blot with a standard ballpoint pen.
11. Coat the remaining protein binding sites on the nitrocellulose by incubating the blot in TSM buffer (50–100 ml per blot) for 6–12 h at room temperature (*see* **Note 6**). Remove the blot from the TSM buffer. Wash the blot four times in quick succession with TS buffer (25–50 ml per wash) and dry the blot on fresh paper towels.

Either before or after coating of the unoccupied protein binding sites, blots can be dried and stored indefinitely in an appropriate container, e.g. disposable resealable food storage bags (1–2 blots/bag).

3.2. Detection of Antibody-Antigen Complexes Using Peroxidase-Coupled Secondary Antibody

1. Place the nitrocellulose blot in a container appropriate for reaction with the antibody. A 15- or 20-lane sheet can be reacted with 15–20 ml of antibody solution in a resealable plastic bag or an open plastic dish of suitable size. A 1- or 2-lane strip can be reacted with 2–5 ml of antibody solution in a disposable 15 ml conical test tube.
2. If the nitrocellulose has been dried, rehydrate it by incubation for a few min in a small volume of TSM buffer.
3. Add an appropriate dilution of antibody to the TSM buffer and incubate overnight (10–15 h) at 4° C with gentle agitation (*see* **Notes 7** and **8**).
4. Remove the antibody solution and save for reuse (*see* **Note 9**).
5. Wash the nitrocellulose (100 ml/wash for each large blot or 15–50 ml/wash for each individual strip) with PBS containing 2 *M* urea and 0.05% NP-40 (three washes, 15 min each) followed by PBS buffer (two washes, 5 min each) (*see* **Note 10**).
6. Add freshly prepared PBS containing 3% milk to the nitrocellulose sheets or strips (*see* **Note 11**). For nitrocellulose sheets (or pooled strips) in resealable plastic bags it is convenient to use a 25 ml volume. Add peroxidase-labeled secondary antibody at a suitable dilution (*see* **Note 12**). Incubate for 90 min at room temperature.
7. Remove the secondary antibody and discard appropriately.

8. Wash the sheets (100 ml/wash for each large blot or each group of pooled strips) with PBS containing 0.05% NP-40 (two washes of 5 min each, two washes of 15 min each, and two washes of 5 min each).
9. Expose blots to enhanced chemiluminescent reagents according to the supplier's protocol.
    a. In the case of Supersignal®, combine equal volumes of the two solutions (e.g., 5 ml of each for a gel containing 20 lanes).
    b. Remove the last wash solution from the blot.
    c. Incubate the blot with the combined enhanced chemiluminescence reagents for 60 sec with gentle agitation.
    d. Remove enhanced chemiluminescence reagent from container (*see* **Note 13**). Remove excess reagent but do not dry the blot.
    e. Seal the bag containing the blot and a minimal amount of enhanced chemiluminescence reagent. Immediately expose to X-ray film (*see* **Notes 14** and **15**).
10. After exposure to X-ray film, rinse the blot three or four times with PBS (25 ml/wash) and store it dry at room temperature.

3.3. Dissociation of Bound Antibodies

1. Place the blot to be stripped in a resealable plastic bag.
2. Add 50 ml of erasure buffer, seal the bag, and incubate in a water bath at 65°C for 30 min with gentle agitation every 5–10 min (*see* **Notes 16-18**).
3. Decant and discard the erasure buffer. Rinse the blot three times with 50–100 ml of TS buffer to remove SDS.
4. To ensure that nonspecific binding sites on the blot are well coated, incubate with TSM buffer for at least 2 h at room temp. with gentle agitation.
5. The blot is ready to be dried and stored or to be incubated with a new antibody as described in **Subheading 3.2**.

3.4. Modification for Dissociation of Antibodies After Autoradiographic Detection

1. Perform steps 1–8 as described in **Subheading 3.2**, substituting 5–10 μCi (^{125}I)-labeled secondary antibody for peroxidase-coupled antibody.
2. Rather than exposing the blot to enhanced chemiluminescence reagents (**Subheading 3.2**, step 9), follow the last wash in PBS/Tween with a 5-min incubation with TSM buffer. This incubation facilitates subsequent removal of antibody and reuse of the blot (*see* **Notes 19** and **20**).
3. After incubating the blot with TSM buffer, immediately dry it between several layers of paper towels. After 5 min, move the blot to fresh paper towels to prevent the nitrocellulose from sticking to the paper towels. When the blot has dried thoroughly, tape it to a solid support (e.g. thin cardboard), wrap it with clear plastic wrap, and subject it to autoradiography. (For details, see ***ref. 34***.)
4. After autoradiography, remove antibodies as indicated in **Subheading 3.3, steps 1–5.**

4. Notes

4.1. Transfer of Polypeptides to Nitrocellulose

1. The method described is for transferring polypeptides after electrophoresis in SDS-containing polyacrylamide gels. Alternative methods have been described for transferring polypeptides after acid-urea gels and after isoelectric focusing (reviewed in ***refs. 2, 3*** and Chapter 61).
2. Choice of solid support for polypeptides: Various solid supports (nitrocellulose, nylon or PVDF) can be used for Western blotting, stripped and reprobed. Use of nitrocellulose and PVDF is illustrated in **Fig. 2**. Nitrocellulose is easy to use and is compatible with a wide variety of staining procedures. With multiple cycles of blotting and erasing, however, nitrocellulose becomes brittle. PVDF membranes are durable, are compatible with a variety of nonspecific protein stains, and are capable of being stripped and reprobed. PVDF membranes, however, require a pre-wetting step in methanol before protein can be transferred. Derivatized nylon has the advantage of greater protein binding capacity and greater durability, but avidly binds many nonspecific protein stains (reviewed in ***refs. 1-3***). The higher binding capacity of nylon is said to contribute to higher background binding despite the use of blocking solutions containing large amounts of protein (reviewed in ***ref. 3***). As illustrated below (see **Fig. 4** later in this chapter), however, antibodies can be more easily dissociated from nylon than from nitrocellulose.
3. Various compositions of transfer buffer have been described (reviewed in ***refs. 1–3***). Methanol is said to facilitate the binding of polypeptides to nitrocellulose, but to retard the electrophoretic migration of polypeptides out of the gel. In the absence of SDS, polypeptides with molecular weights above 116 kDa do not transfer efficiently. Low concentrations of SDS (0.01 to 0.1%) facilitate the transfer of larger polypeptides, but simultaneously increase the current generated during electrophoretic transfer, necessitating the use of vigorous cooling to prevent damage to the transfer apparatus.
4. Alternative staining procedures (reviewed in ***refs. 1-3***) utilize Coomassie blue, Ponceau S, Amido black, India drawing ink, colloidal gold, or silver. A highly sensitive technique utilizing eosin Y has also been described ***(35).***
5. A washing step in acidified alcohol is probably essential to immobilize the polypeptides on the nitrocellulose ***(3, 31, 32)***. The fast green staining procedure satisfies this requirement. Polypeptides are observed to elute from nitrocellulose under mild conditions if a wash in acidified alcohol is omitted ***(32, 36)***.
6. Various protein solutions have been utilized to block unoccupied binding sites on nitrocellulose (reviewed in ***refs. 2, 3***). These include 5–10% (w/v) powdered dry milk, 3% (w/v) bovine serum albumin, 1% (w/v) hemoglobin,

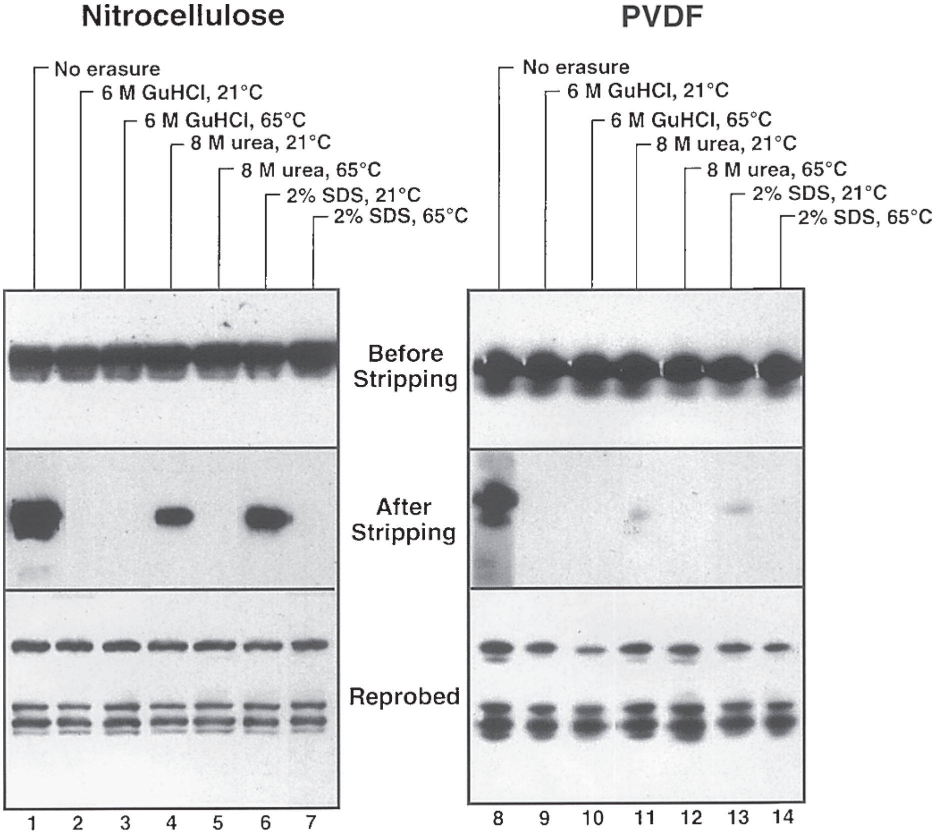

Fig. 2. Conditions for dissociating antibodies from nitrocellulose or PVDF after immunoblotting with enhanced chemiluminescence detection. Replicate samples containing whole cell lysates prepared from 3 × 10^5 K562 human leukemia cells were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose (lanes 1–7) or PVDF (lanes 8–14). After unoccupied binding sites were blocked by incubation with TSM buffer, blots were incubated with a chicken polyclonal antiserum that reacts with the nucleolar protein B23 followed by peroxidase-labeled goat anti-chicken IgG. After detection with enhanced chemiluminescence reagents (upper panels), the blots were dried. The indicated strips were then incubated for 30 min at 21°C or 65°C with 62.5 mM Tris-HCl (pH 6.8 at 21°C) containing 100 mM 2-mercaptoethanol and 6 M guanidine hydrochloride (GuHCl), 8 M urea, or 2% (w/v) SDS as indicated. At the completion of the incubation, the strips were washed four times with PBS, recoated for 6 h in TSM buffer, and dried overnight. The strips were then reprobed with peroxidase-coupled goat anti-chicken IgG (**Subheading 3.2, steps 6–9**) to detect primary antibodies that were still bound (middle panels). After being dried again, strips were probed with rabbit anti-protein kinase Cδ followed by peroxidase-coupled goat anti-rabbit IgG to confirm that the cellular polypeptides remained bound to the strips (lower panels). Note that the efficacy of various treatments at removing antibodies varied depending upon the solid support, the buffer utilized for stripping, and the

and 0.1% (w/v) gelatin. Although the choice of protein can affect antibody binding (e.g., **Fig. 4C**), antibodies have been successfully stripped from blots of coated with any of these solutions ***(30)***.

4.2. Formation of Antigen-Antibody Complexes

7. No guidelines can be provided regarding the appropriate dilution of antibody to use. Some antisera are useful for blotting at a dilution of >1:20,000. Other antisera yield detectable signals only when used at a dilution of 1:5 or 1:10. When attempting to blot with an antiserum for the first time, it is reasonable to try one or more arbitrary concentrations in the range of 1:10 to 1:500. If a strong signal is obtained at 1:500, further dilutions can be performed in subsequent experiments.
8. Different investigators incubate blots with primary antibodies for different lengths of time and at different temperatures. Preliminary studies with some of our antibodies have indicated that the signal intensity is greater when blots are incubated with antibody overnight compared to 1–2 h at room temperature. Incubation at 4°C is specifically recommended for many currently available anti-phosphoepitope antisera, presumably because of instability of the affinity purified antibodies; and the 4°C incubation also yields good results with other antisera.
9. Most diluted antibody solutions can be reused multiple times. They should be stored at 4°C after additional aliquots of penicillin/streptomycin and sodium azide have been added. Some workers believe that the amount of nonspecific (background) staining on Western blots diminishes as antibody solutions are reutilized. Antibody solutions are discarded or supplemented with additional antibody when the intensity of the specific signal begins to diminish.
10. Choice of wash buffer after incubation with primary antibody: 2 M urea is included in the suggested wash buffer to diminish nonspecific binding. Alternatively, some investigators include a mixture of SDS and nonionic detergent (e.g., 0.1% (w/v) SDS and 1% (w/v) Triton X-100) in the wash buffers. For antibodies with low avidity (especially monoclonal antibod-

Fig. 2 (continued): temperature applied. After enhanced chemiluminescence detection, all of these denaturing buffers removed the anti-B23 antibodies at 65°C. At 21°C, urea and SDS left substantial amounts of antibody bound to nitrocellulose (middle panel, lanes 4 and 6) and small but detectable amounts of antibody bound to PVDF (middle panel, lanes 11 and 13). In each case, the stripping procedure had little or no effect on subsequent blotting with another antiserum (bottom panels).

ies and anti-peptide antibodies), the inclusion of 2 M urea or SDS might diminish the signal intensity. These agents are, therefore, optional depending upon the properties of the primary antibody used for blotting.

11. It is important to avoid sodium azide when using horseradish peroxidase-coupled antibodies, as the azide inhibits peroxidase ***(23, 24)***. For this reason, adding milk to PBS immediately before use of each aliquot of this buffer is advisable.
12. The concentration secondary antibody is determined empirically. Most suppliers recommend a dilution for their reagents, typically to the 0.1–0.5 μg/ml range.
13. The same enhanced chemiluminescence reagent can be transferred from one blot to another to develop several blots simultaneously.
14. The length of exposure required to give a good signal varies depending on the abundance of the antigen, the quality of the primary and secondary antibodies, and the nature of the solid support utilized. One convenient way to proceed is to expose each blot for an arbitrary time (e.g., 2 min) and then increase or decrease the exposure time based on the results of the trial exposure.
15. The signal obtained with some chemiluminescence reagents decays with a half-time of 30–60 min. With these reagents, it is important to expose film promptly after treating blots. Other chemiluminescence reagents continue to generate a strong signal for many hours.

4.3. Dissociation of Antibodies after Chemiluminescent Detection

16. Choice of erasure buffer: Experiments showing the effect of various erasure buffers on removal of primary antibodies are illustrated in **Fig. 2**. After detection by enhanced chemiluminescence, bound antibodies can be solubilized using 6 M guanidine hydrochloride, 8 M urea, or 2% SDS. None of these treatments elutes significant amounts of antigen from nitrocellulose (lower panel, **Fig. 2**). After enhanced chemiluminescent detection, antibodies appear to be more easily removed from PVDF than from nitrocellulose (cf. lanes 6 and 13 in **Fig. 2**).
17. a. Temperature of incubation: When blotting is performed after immobilization of polypeptides on nitrocellulose, complete removal of antibodies by either 8 M urea or 2% SDS requires heating to ≥ 50°C for 30 min ***(ref. 30***; see also **Fig. 2**, lanes 4–7).
    b. Length of incubation. When blotting is performed on nitrocellulose, complete dissociation of radiolabeled antibodies at 70°C requires a minimum of 20 min incubation with erasure buffer ***(30)***. Incubation times for removal of antibodies after chemiluminescent detection have not been systematically investigated.

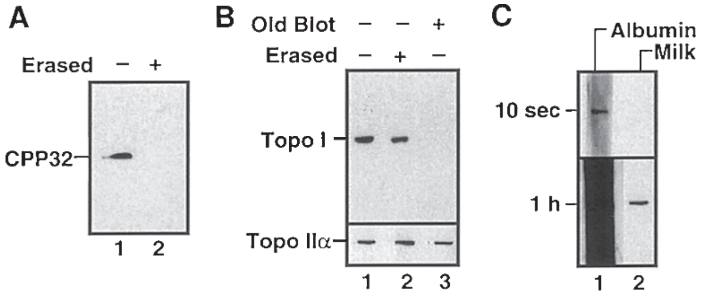

Fig. 3. Effect of various treatments on antigen recognition. (**A**) Replicate aliquots containing polypeptides from 2 × 10^5 K562 cells were separated by SDS-PAGE, transferred to nitrocellulose, and stained with fast green FCF (**Subheading 3.1, steps 1–11**). Strip 2 was then treated as described in Section 3.3 (steps 1–5) to simulate an erasure procedure. Strips were blocked with TSM buffer and blotted with a mouse monoclonal IgG recognizing CPP32/procaspase-3. The epitope recognized by this antibody was destroyed by erasure (lane 2). In contrast, many other epitopes are not (e.g., **Fig. 2,** lower panels). (**B**) Replicate gel lanes containing identical amounts of protein from a single batch of HL-60 leukemia cell lysate were transferred to nitrocellulose one week (strips 1 and 2) or three years (strip 3) prior to blotting. Strip 2 was treated as described in **Subheading 3.3, steps 1–5**, to simulate an erasure procedure. After unoccupied binding sites were reblocked by incubation with TSM buffer, the strips were incubated with the monoclonal antibodies C-21, which recognizes DNA topoisomerase I (upper panel), or Ki-S1, which recognizes DNA topoisomerase IIα (lower panel). After washing and incubation with peroxidase-coupled secondary antibodies, blots were subjected to chemiluminescent detection. The epitope recognized by antibody C-21 was damaged by prolonged storage (lane 3, upper panel) but not by the erasure procedure (lane 2, upper panel). In contrast, the epitope recognized by antibody Ki-S1 was unaffected by either manipulation. **C.** Effect of blocking solution on reactivity. Replicate gel lanes containing protein from 3 × 10^5 HL-60 cells were blocked with TS buffer containing 3% albumin (strip 1) or 10% milk (strip 2), then reacted with antibody Ki-S1 diluted 1:1000 in the corresponding protein solution. After reaction with peroxidase-coupled anti-mouse IgG diluted in the corresponding protein solution, blots were treated with luminol and exposed to Kodak XAR-5 film for 20 seconds (upper panel) or 1 h (lower panel). The signal was more rapidly detected when albumin was used as the blocking protein, but the background was much cleaner using milk as a blocking reagent.

18. Although most epitopes are resistant to the erasure procedure ***(29, 30),*** epitopes recognized by an occasional monoclonal antibody are destroyed (**Fig. 3A**, lane 2). Observations from our laboratory also indicate that certain epitopes are lost upon prolonged storage of blots (**Fig. 3B**, lane 3). There does not appear to be any relationship between the loss of epitopes upon blot storage and the damage of epitopes during the erasure procedure.

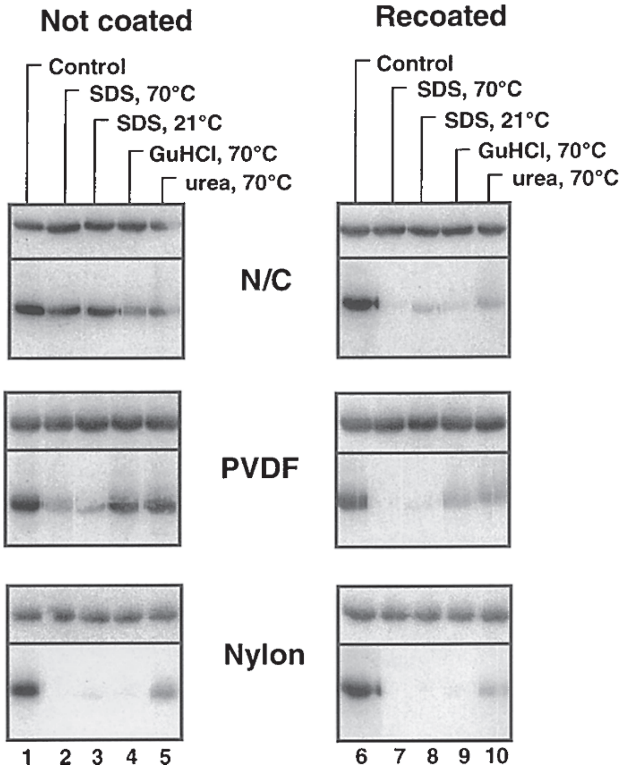

Fig. 4. Conditions for dissociating radiolabeled antibodies after immobilization of polypeptides on various solid supports. Replicate samples containing 2 × 10^6 rat liver nuclei were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose, PVDF, or Nytran nylon sheets as indicated (*see* **Note 2**). After staining with fast green FCF and treatment with TSM buffer to coat unoccupied protein binding sites (**Subheading 3.1, steps 8–11**), blots were incubated with chicken polyclonal antiserum that reacts with the nuclear envelope polypeptide lamin B_1 (39) followed by (^{125}I)-labeled rabbit anti-chicken IgG (**Subheading 3.4, step 1**). Half of each blot (lanes 6–10) was recoated with milk-containing buffer for 5 min prior to drying (**Subheading 3.4, step 2**) and the other half (lanes 1–5) was dried without being recoated with protein. After autoradiography confirmed that the signals in all lanes of a given panel were comparable (upper panels), samples were incubated for 30 min at the indicated temperature with 50–62.5 mM Tris-HCl (pH 6.8) containing 100 mM 2-mercaptoethanol and one of the following denaturing agents: 2% (w/v) SDS, 6 M guanidine hydrochloride (GuHCl) or 8 M urea. Following this erasure procedure, strips were subjected to autoradiography again (bottom panels). In each case, untreated strips (lanes 1 and 6) served as controls. This analysis led to several conclusions. First, recoating with milk prior to drying (**Subheading 3.4, step 2**) did not affect the amount of radiolabeled antibody initially bound to the blots (cf. lanes 1 and 6). Second, the efficacy of various treatments at removing these antibodies varied depending upon the solid support. For nitrocellulose or PVDF, coating of the blots with protein prior to drying facilitated the subsequent dissociation

4.4. Removal of Antibodies After Autoradiographic Detection

19. The major modification suggested for removal of radiolabeled secondary antibodies is an incubation of blots with protein-containing buffer immediately prior to drying. For reasons that are unclear, this step appears to be essential for efficient dissociation of certain antibodies from nitrocellulose or PVDF paper after incubation with radiolabeled secondary antibodies **(Fig. 4)**, but not after incubation withperoxidase-coupled antibodies **(Fig. 2)**.
20. (^{125}I)-labeled protein A can be substituted for radiolabeled secondary antibody. Protein A, however, can bind to the immunoglobulins present in milk, causing a high background on the blot. Therefore, when (^{125}I)-labeled protein A is to be used, milk should not be utilized to block unoccupied binding sites (Section 3.1, step 11), nor as a diluent for antibodies (**Subheading 3.2.,** steps 2, 3 and 6). Instead, bovine serum albumin, hemoglobin, or gelatin should be considered (*see* **Note 6**).

4.5. General Notes

21. Some blotting procedures involve deposition of chromogenic reaction products directly on immunoblots by enzyme-coupled secondary antibodies ***(5, 37, 38)***. The technique described above is not useful for removing colored peroxidase reaction products (e.g., diaminobenzidine oxidation products) from blots.
22. A modification of the techniques described in **Subheading 3.2** allows the detection of glycoproteins by radiolabeled lectins. For this application, blots would be coated with albumin or gelatin, reacted with radiolabeled lectin (**Subheading 3.2.,** Steps 6–8), and recoated with albumin or gelatin (**Subheading 3.4.,** Steps 2 and 3) prior to drying. After autoradiography, the radiolabeled lectin would be solubilized in warm SDS under reducing conditions **(Subheading 3.3)**.

Fig. 4 (continued): of the anti-lamin B antibodies. In both cases, SDS-containing buffer (lanes 7,8) was more effective than guanidine hydrochloride (lane 9) or urea (lane 10). For nylon, SDS was again slightly more effective than urea at dissociating the antibodies (cf. lanes 2 vs. 5 and 7 vs. 10). Interestingly, when SDS-containing erasure buffer was used, it was not necessary to recoat nylon with protein prior to drying for autoradiography (cf. lanes 2 and 7). This was in contrast to nitrocellulose or PVDF, which were examined in the same experiment.

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The Use of Quantum Dot Luminescent Probes for Western Blot Analysis

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1. Introduction

Western blot analysis ***(1,2)*** is one of the most widely used methods for analyzing specific protein targets using colorimetric or chemiluminescent substrates ***(3)***. However, the reporting labels that are typically used for protein detection are unsuitable for simultaneous detection of multiple targets. In order to detect different proteins the blot is usually stripped and reprobed, an approach that is time- and labor-intensive, and often leads to loss of immobilized proteins from the blot. Detection of multiple antigens on a single protein blot without stripping off prior antibodies is possible, but requires sequential reactions ***(4)***. More recent approaches employ multiple fluorescent probes made from small organic dye molecules (***(5)*** and references therein), but such probes have several limitations, described below. The use of QD luminescent labels has the potential to eliminate most of these problems.

QDs are semiconductor nanoparticles (e.g. CdSe, InP, InAs) with diameters in the range of 2 to 10 nm whose fundamental physical properties are influenced by quantum confinement effects ***(6)***. QDs exhibit absorption and emission peaks that progressively shift to longer wavelengths with increasing particle size. For applications as fluorescent markers, the relevant quantum confinement effect is the tuning of the semiconductor band gap and hence the color of the QD emission achieved simply by changing the size of the particle. In this way, the luminescence wavelength of QDs can be tuned from the infrared to the ultraviolet ***(7)***. **Fig. 1** shows the photoluminescence of CdSe nanocrystals spanning the visible spectrum with particles ranging in diameter from 2 to 6 nm.

QDs have significant advantages compared to standard fluorescent organic dyes. For example, dyes have narrow absorption bands, hence it is difficult

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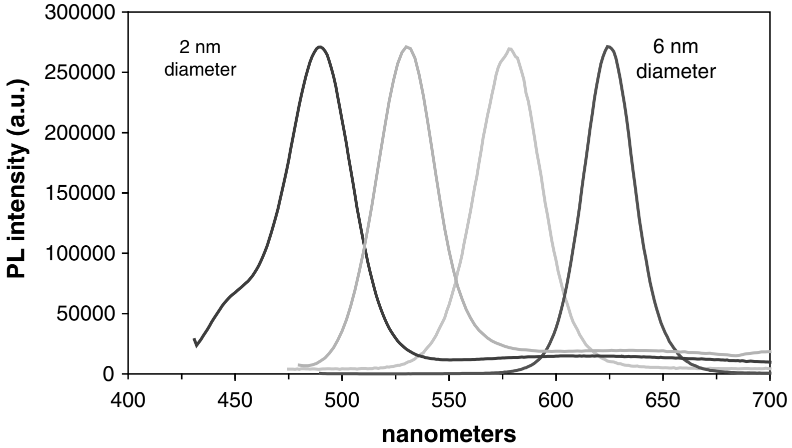

Fig. 1. Photoluminescence of CdSe nanocrystals with diameters ranging from 2 to 6 nm.

to excite several colors with a single excitation source. Moreover, the broad spectral overlap between the emissions of dyes requires complex mathematical analysis of the data. In contrast, QDs have a narrow, tunable, symmetric emission spectrum, permitting a larger number of probes within a detectable spectral region. Different-size populations of QDs can be excited with a single light source, making possible the development of simpler and more cost-effective instrumentation for multiplex detection of biomolecules. QDs are considerably more stable against photobleaching than organic dyes ***(8, 9)***. This is an important property, particularly for imaging applications, where the high photostability of QDs allows real-time monitoring of intracellular processes over longer periods of time ***(10, 11)***. QDs exhibit large Stokes shifts (the difference between the maximum absorbance and emission wavelengths). This property allows the target signals to be separated clearly from autofluorescence, and enables the entire emission spectra to be collected, in contrast to the use of fluorescent proteins, e.g., green fluorescent protein. The biological applications of QDs have been reviewed recently ***(12, 13)***.

In this chapter we describe a bioconjugation method that permits the facile generation of QD-based probes for multiplex detection of proteins in Western blots ***(14)***. The strategy for immobilization of antibodies to QDs utilizes the IgG-binding "Z" domain, which is based on the "B" domain of *Staphylococcus aureus* protein A ***(15, 16)***. The Z affinity tag (6.5 kDa) is highly specific for its ligand, IgG Fc, and can easily be purified by affinity chromatography using IgG-Sepharose. It has been shown ***(17)*** that the divalent ZZ domain exhibits ten times higher affinity (K_{aff} ~3×10^8 M^{-1}) for its IgG ligand, than the monovalent Z domain (K_{aff} ~2×10^7 M^{-1}). We engineered a ZZ protein fused to a peptide that is biotinylated *in vivo* ***(18)***, followed by a hexahistidine tag. The biotinylated ZZ tag was produced in bacteria, purified on a monomeric avidin or Ni^{++}-NTA matrix, and coupled to streptavidin-coated QDs, thus enabling biospecific conjugation of any antibody to the functionalized QDs (**Fig. 2**).

Fusion proteins containing a specific biotinylation motif are biotinylated *in vivo* by biotin protein ligase, the product of the *birA* gene, which recognizes a 75-amino acid residue sequence as its biotinylation substrate ***(19)***. Such proteins can be affinity-purified using immobilized monomeric avidin and mild elution conditions that do not damage the biotinylated protein. This approach is advantageous over *in vitro* biotinylation, an indiscriminate process that may adversely affect the structure and/or function of the modified protein through the modification of critical lysine residues. Schatz ***(18)*** identified a consensus 13-residue peptide that is biotinylated *in vivo* at an invariant lysine residue. This sequence can replace the larger biotinylation signal, and has been used successfully for the purification of fusion proteins (e.g., ***(20)***). A potential disadvantage of the affinity chromatographic purification of *in vivo* biotinylated proteins is the need to remove free biotin from the cell lysate in order to avoid interference with the purification of the target protein. In addition, biotinylated host proteins may possibly be copurified with the target protein. These potential complications are easily avoided by including an additional affinity tag that facilitates protein purification by an alternative method prior to the use of avidin-chromatographic

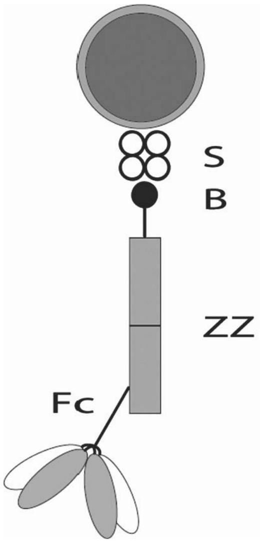

Fig. 2. Schematic representation of the QD-based probe. There are approximately 5–10 streptavidin molecules on the QD surface. S, streptavidin; B, biotin; ZZ, head-to-tail dimerized Z domain derived from protein A. The various probe components are not drawn to scale. Reprinted with permission from the publisher (14).

media ***(21)***. We included a $(His)_6$ tag located downstream of the biotinylation sequence to facilitate purification of the biotinylated ZZ using a Ni^{++}-NTA matrix. The ZZ tag was purified from *E. coli* using monomeric avidin as well as Ni^{++}-NTA, and analyzed by Western blot probed with an avidin-alkaline phosphatase conjugate. The results confirmed the presence of biotinylated ZZ protein, with no evidence of additional biotinylated bands, irrespective of the purification method ***(14)***.

The ZZ tag-derivatized surface of QDs enables facile conjugation to secondary (or primary) antibodies, eliminates the labor and expense of custom-labeling secondary antibodies with different enzymes, biotin, and other tags, and avoids the potential non-availability of secondary antibodies with the desired label. The only requirement for specificity of protein detection in our system is the matching of primary and secondary antibodies. This too can be avoided by directly conjugating primary antibodies to ZZ-functionalized QDs, although, in this case, the sensitivity of detection may be attenuated. The efficiency of ZZ biotinylation may also be enhanced by using a more efficient recognition sequence for *in vivo* biotinylation ***(22)***. An alternative approach for labelling QDs employs the full-length protein A. The advantage of our method is that the ZZ peptide does not bind the Fab region of Ig molecules, in contrast to all individual domains of protein A ***(23,24)***. This offers the advantage of a more uniform binding to antibodies (via the Fc region), leaving the Fab domain free to bind its target, unlike direct QD-antibody binding, which sterically blocks many of the antibody active sites. Thus, our QD bioconjugation strategy could be useful for quantitative assays.

2. Materials

2.1. Construction of pEZZ-B6H

1. Phagemid pEZZ18 (Amersham Biosciences, Piscataway, NJ).
2. TAE buffer: 40 m*M* Tris, 5 mM sodium acetate, 1 m*M* EDTA, pH 7.8.
3. GeneClean II Kit (Q-Biogene, Carlsbad, CA).
4. Synthetic oligonucleotides (Qiagen, Valencia, CA).
5. Rapid DNA Ligation Kit (Roche, Indianapolis, IN).
6. HiSpeed Plasmid Maxi Kit (Qiagen).
7. *E. coli* BL21 competent cells (Novagen, Madison, WI).
8. *E. coli* AVB101 competent cells (Avidity, Denver, CO).

2.2. Expression

1. LB medium: 2.5 g Bacto-Tryptone, 1.25 g Bacto-Yeast Extract, 2.5 g NaCl, pH 7.2–7.4, per liter H_2O.
2. Ampicillin (Sigma Chemical Company, St. Louis, MO).
3. Chloramphenicol (Sigma).
4. Biotin (Pierce, Rockford, IL).

5. Isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma).

2.3. Purification of ZZ-B6H Protein

1. Phenylmethylsulfonyl fluoride (PMSF) (Roche).
2. Sucrose buffer: 0.5 *M* sucrose, 0.1 *M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
3. 5 m*M* $MgSO_4$.
4. 0.45 µm nitrocellulose membrane filter (Nalgene, Rochester, NY).
5. Monomeric avidin column (Pierce).
6. Slide-a-Lyzer Cassettes (Pierce).
7. PBS: 0.01 *M* KH_2PO_4, 0.1 *M* Na_2HPO_4, 1.37 *M* NaCl, 0.027 *M* KCl.
8. Bovine serum albumin (Sigma).
9. BCA Protein Assay (Pierce).
10. Agarose-Ni^{++}-NTA chromatography column (Qiagen).
11. Wash buffer: 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 20 m*M* imidazole, pH 8.0.
12. Elution buffer: 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 250 m*M* imidazole, pH 8.0.
13. Amicon Ultra-15 centrifugal filters (5,000 nominal molecular weight limit, NMWL) (Millipore, Bedford, MA).

2.4. QD Probe Assembly

1. Streptavidin-functionalized CdSe/ZnS core/shell QDs of different size and emission wavelength (Invitrogen, Carlsbad, CA). QDs are provided in a solution which is 2 mM total Cd concentration. The CdSe core is encapsulated in a shell of ZnS and the polymer shell, which may prevent release of free Cd. However, QDs should be considered to be toxic and be disposed of accordingly (http://probes.invitrogen.com/products/qdot/faq.html).
2. YM-100 Microcon filters (100,000 NMWL) (Millipore).
3. Human apolipoprotein AI (EMD Biosciences, San Diego, CA).
4. Firefly luciferase (EMD Biosciences).
5. TBS-T buffer: 100 m*M* Tris-HCl pH 7.4, 150 m*M* NaCl, 0.1% (v/v) Tween-20.
6. Rabbit anti-apoAI (EMD Biosciences).
7. Goat anti-luciferase (Rockland Immunochemical, Gilbertsville, PA).

2.5. Western Blot

1. Electrophoresis apparatus (Invitrogen).
2. 1X LDS Sample Buffer (Invitrogen).
3. 4–12% NuPAGE Bis-Tris gels (Invitrogen).
4. 1X MES buffer (Invitrogen).
5. PVDF membranes (Immobilon-P) (Millipore).
6. Panther Semidry Electroblotter (model HEP-1) and buffer reagents (Owl Separation Systems, Portsmouth, NH).
7. SuperBlock blocking buffer (Pierce).
8. SeaBlock reagent (Pierce).
9. Simply Blue Safe Stain (Invitrogen).
10. Avidin-alkaline phosphatase (AP) conjugate (Pierce).

11. NBT/BCIP substrate (Pierce).
12. Biotinylated protein A (Pierce).

2.6. Image capture and analysis

1. UV light transilluminator (Fotodyne, Hartland, WI).
2. Minolta digital camera (model A1)
3. NIH Image J software (http://rsb.info.nih.gov/ij).

3. Methods

3.1. Construction of pEZZ-B6H

The phagemid pEZZ18 contains the protein A signal sequence (S), two Z domains, and a polylinker site located 3′ to the ZZ domain ***(16)***. Expression is controlled by the protein A constitutive promoter.

1. The polylinker of pEZZ18 is digested with *Eco*RI and *Pst*I, and the large vector fragment is purified from a 0.8% agarose gel in TAE buffer (40 m*M* Tris, 5 m*M* sodium acetate, 1 m*M* EDTA, pH 7.8) using the GeneClean II Kit.
2. Two synthetic complementary oligonucleotides, 115 and 107 bases long, respectively, are designed to encode the *in vivo* biotinylation signal sequence ***(18)*** and six histidine residues followed by two stop codons, including sequences to regenerate the *Eco*RI, *Bam*HI, and *Pst*I restriction sites (underlined): 5′ -AAT TCG GGC AGC GCG AGC AGC CTG CGC CAG ATT CTG GAT AGC CAG AAA ATT GAA TGG CGC AGC AAC GCG GGC GGC AGC GGC CAT CAT CAT CAT CAT CAT TAA TAA GGA TCC TGC A-3′ and 5′ -GGA TCC TTA TTA ATG ATG ATG ATG ATG ATG GCC GCT GCC GCC CGC GTT GCT GCG CCA TTC AAT TTT CTG GCT ATC CAG AAT CTG GCG CAG GCT GCT CGC GCT GCC CG-3′ . This DNA sequence is designed using the codon usage frequency for *E. coli* K12 (Codon Usage Database, http://www.kazusa.or.jp/codon).
3. The oligonucleotides are separately resuspended in 10 m*M* Tris-HCl, pH 8.0, 10 m*M* NaCl, and annealed by mixing equimolar amounts and heating to 100°C followed by slow cooling to 22°C.
4. The linearized vector is ligated to the double-stranded oligonucleotide using the Rapid DNA Ligation Kit. The resulting plasmid pEZZ-B6H (**Fig. 3**) is transformed into *E. coli* BL21 competent cells, and the plasmid is prepared using a HiSpeed Plasmid Maxi Kit. The plasmid is also transformed into AVB101 competent cells, which harbor a chloramphenicol-resistant plasmid pJS169 encoding the BirA biotin ligase gene.

3.2. Expression

1. *E. coli* AVB101 cells harboring plasmids pJS169 and pEZZ-B6H are grown in 250 ml LB medium containing 100 µg/ml ampicillin and 10 µg/ml chloramphenicol in baffled flasks at 37°C, 90 rpm, to an OD_{600nm} 0.5.

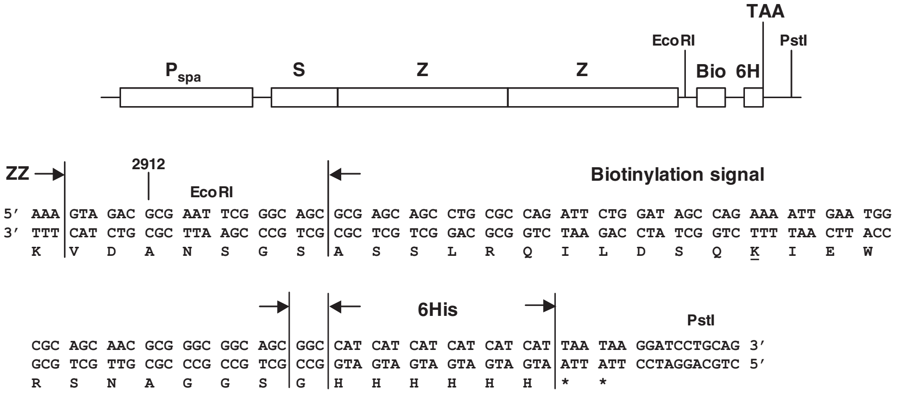
TAA
EcoRI
PstI
P_{spa}
S
Z
Z
Bio
6H
ZZ
2912
EcoRI
Biotinylation signal
5′ AAA GTA GAC GCG AAT TCG GGC AGC GCG AGC AGC CTG CGC CAG ATT CTG GAT AGC CAG AAA ATT GAA TGG
3′ TTT CAT CTG CGC TTA AGC CCG TCG CGC TCG TCG GAC GCG GTC TAA GAC CTA TCG GTC TTT TAA CTT ACC
K V D A N S G S A S S L R Q I L D S Q K I E W
6His
PstI
CGC AGC AAC GCG GGC GGC AGC GGC CAT CAT CAT CAT CAT CAT TAA TAA GGATCCTGCAG 3′
GCG TCG TTG CGC CCG CCG TCG CCG GTA GTA GTA GTA GTA GTA ATT ATT CCTAGGACGTC 5′
R S N A G G S G H H H H H H * *

Fig. 3. Configuration and DNA sequence of selected genetic elements in vector pEZZ-B6H for production of the ZZ tag in *E. coli.* Pspa, promoter of the Staphylococcal protein A; S, signal peptide; ZZ, IgG-binding dimeric domain derived from protein A; Bio, in vivo biotinylation tag; 6H, hexahistidine tag; TAA (*), termination codon. The boundaries of the ZZ moiety, the biotinylation, and hexahistidine tags are shown, and the single lysine residue (K) that is biotinylated is underlined. The number 2912 refers to the nucleotide position in the parental plasmid pEZZ18 (GeneBank accession number M74186). Reprinted with permission from the publisher (14).

2. The medium is then supplemented with 50 µM biotin and 1 mM IPTG in order to induce the expression of the *birA* gene. The cells are further grown for 5 h.

3.3. Protein extraction and purification

1. The cells are harvested by centrifugation at 5,000g for 15 min at 4°C, and the ZZ tag (M.W. ~17,000) is extracted from the periplasm by the osmotic shock procedure ***(25)***, modified as follows:
2. The cell pellet is resuspended in 1/20 original culture volume of ice-cold sucrose buffer.
3. PMSF is added to 1 m*M* final concentration, and the suspension is stirred slowly at room temperature for 10 min.
4. The cells are collected by centrifugation at 15,000 g for 10 min at 4°C.
5. The cell pellet is resuspended in 12 ml ice-cold 5 m*M* $MgSO_4$, and stirred slowly for 10 min at 4°C.
6. The shocked cells are centrifuged at 15,000g for 10 min at 4°C, and the supernatant is filtered through a 0.45 µm nitrocellulose membrane filter.
7. The ZZ tag is purified on a monomeric avidin column using 2 m*M* biotin in PBS for elution.
8. Protein fractions identified by polyacrylamide gel electrophoresis (PAGE) and Western blotting are pooled and dialyzed at 4°C in Slide-a-Lyzer Cassettes (Pierce) against three changes of PBS.

9. The concentration of the ZZ peptide is determined using the BCA Protein Assay and bovine serum albumin as the standard.
10. Alternatively, ZZ is purified on an agarose-Ni^{++}-NTA chromatography column. The osmotic shock supernatant (12 ml) is loaded onto 4 ml Ni^{++}-NTA resin that is equilibrated with 40 ml wash buffer and ZZ is eluted using 20 ml elution buffer.
11. Fractions (1.5 ml) are analyzed by PAGE and Western blotting. Fractions of interest are pooled and concentrated in PBS via repeated centrifugation (5,000 g, 30 min in fixed angle rotor) using Amicon Ultra-15 centrifugal filter devices (5,000 NMWL).

3.4. Western Blotting of the Biotinylated ZZ Protein

1. ZZ samples (18 µl, ~2 µg) are prepared in 1X LDS Sample Buffer in a total volume of 25 µl, incubated at 70°C for 10 min, and loaded onto 4–12% NuPAGE Bis-Tris gels and electrophoresed at 200 V in 1X MES buffer under non-reducing conditions.
2. Gels are stained by immersing in Simply Blue Safe Stain (Invitrogen) for 60 min and destained in water.
3. The protein bands are electrotransferred to PVDF membranes (Immobilon-P) using the model HEP-1 Panther Semidry Electroblotter and buffer reagents according to the manufacturer's instructions (Owl). Transfer is carried out for 1 h at 1.6 mA/cm^2 (100 mA).
4. After sandwich disassembly the membrane is incubated in SuperBlock blocking buffer (Pierce) for 1 h at 22°C with gentle agitation, or stored in blocking buffer overnight at 4°C.
5. The gel is stained in order to confirm protein transfer.
6. The membrane is incubated for 2 h in blocking buffer containing a 1:1,000 dilution of avidin-alkaline phosphatase (AP) conjugate, and washed three times 10 min each with blocking buffer.
7. The membrane is incubated in NBT/BCIP substrate until bands indicating the presence of ZZ-bound avidin-AP are visible. The positive control lane contains 2 µg biotinylated protein A. The negative control lane contains 2 µg lysozyme.

3.5. QD Probe Assembly

1. Streptavidin-coated CdSe/ZnS core/shell QDs of different size and emission wavelength are coupled to primary antibodies via the ZZ tag, as follows:
2. QD aliquots (10–30 µl of 1 µM stock) are mixed with varying concentrations of dialyzed ZZ peptide in a final volume of 500 µl SeaBlock reagent, and rocked at room temperature for 60 min.
3. The mixture is then centrifuged at 14,000 g for 12 min in YM-100 Microcon filters (100,000 NMWL) in an Eppendorf microcentrifuge, and 450 µl SeaBlock reagent was added to the retentate, followed by centrifugation at 14,000 g for 12 min.
4. The filter device is inverted into a new tube and centrifuged at 1,000g for 3 min to recover the purified probe. Most of the QD probe is typically found in the retentate, with a very small amount of QDs present in the filtrate, as monitored with a hand-held long-wavelength UV lamp.

5. Each purified QD-ZZ conjugate (50 µl) is mixed with 10 µl primary or secondary antibody in a total volume of 500 µl Seablock and rocked at room temperature for 60 min.
6. The probe volume is adjusted to 10 ml (or 5 ml when using two probes together) using SeaBlock, followed by immunodetection.

3.6. Western blotting of test proteins

1. Proteins (human apolipoprotein AI and firefly luciferase) are electrophoresed and electrotransferred to PVDF membranes as described above.
2. The membranes are washed for 5 min in TBS-T and then blocked with SuperBlock or SeaBlock blocking buffer for 1 to 2 h with gentle agitation, or stored overnight at 4°C in blocking buffer.
3. The membranes are then incubated for 1 h with a 1,000-fold dilution of the primary antibodies in blocking buffer (rabbit anti-apoAI, Calbiochem; and goat anti-luciferase, Rockland Immunochemical).
4. The membranes are washed with TBS-T three times for 5 min each, and incubated for 1 to 2 h with QD_{565}-ZZ or QD_{655}-ZZ nanoparticles conjugated to secondary antibody and purified as described above.
5. The blots are then sequentially washed three times for 5 min each using TBS-T, and two times 3 min each using TBS.
6. The proteins are probed alone (**Fig. 4B** and **C**) and in combination (**Fig. 4D**), in order to confirm the specificity of each antibody. The negative-control Western blot is incubated in hybridization buffer without primary antibody. Protein bands are visualized under long-wavelength UV irradiation.
7. Images are captured using a model A1 Minolta digital camera and merged into false-color images using NIH Image J software (http://rsb.info.nih.gov/ij) which is in the public domain.

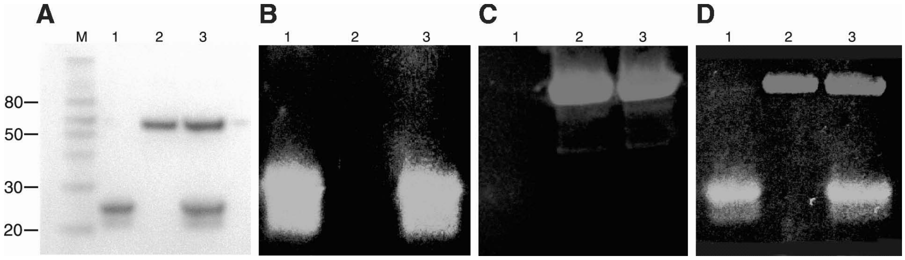

Fig. 4. Western blots of apolipoprotein AI and luciferase. (**A**) PAGE. (M) markers; (1) apoAI (28,330 M.W.); (2) luciferase (62,000 M.W.); (3) both proteins. (B-D): Western blots probed with anti-apoAI-QD565, green color (**B**); anti-luciferase-QD655, red color (**C**); anti-apoAI-QD565 plus anti-luciferase-QD655 (**D**). Control blots were probed with QD-ZZ probes minus primary antibody (not shown). The Western blots were photographed separately, hence band migration appears to be slightly different in different blots. Reprinted with permission from the publisher (**14**).

4. Notes

1. A list of frequently asked questions about QD technical background and troubleshooting tips may be found on the manufacturer's web site: http://probes.invitrogen.com/products/qdot/faq.html
2. QDs fluoresce brightly under a hand-held ultraviolet lamp (long wavelength). This provides a convenient method of monitoring the various experimental steps, e.g., filtration.
3. The detection of luminescent signals may be enhanced by using filters. A list of appropriate and optimal filters is available at: http://probes.invitrogen.com/products/qdot/filters.html
4. A high level of endogenous biotin in the sample may produce high nonspecific background. In this case, the sample may be blocked with BSA or animal serum which will decrease nonspecific binding of both antibodies and QD-streptavidin conjugates. Primary and secondary antibodies should preferably be diluted in the blocking buffer. Endogenous biotin in tissues may be blocked using an Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA).

Acknowledgments

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The Use of Infrared Fluorescent Dyes in Quantitative Immunoblotting

Ching-Hui Yang, Christopher Kasbek, and Harold A. Fisk

1. Introduction

Centrosomes are microtubule organizing centers that regulate assembly of the mitotic spindle apparatus ***(1)***. Because excess centrosomes generate aberrant mitotic spindles, the duplication of centrosomes must be tightly controlled ***(2)***. Centrosome duplication requires the activity of the Mps1 protein kinase ***(3)***, and Mps1 levels are regulated by proteasome-mediated degradation ***(4)***. Mps1 is a Cdk2 substrate, and phosphorylation of Mps1 by Cyclin-dependent kinase 2 (Cdk2) prevents the degradation of Mps1 as part of the mechanism restricting centrosome duplication within the cell cycle ***(4, 5)***. Accordingly, inhibition of Cdk2 activity with the small molecule roscovitine, or depletion of Cdk2 activity with cyclin A- or Cdk2-specific siRNAs, all lead to the loss of Mps1 from centrosomes and prevent centrosome duplication. However, these different means of inhibiting Cdk2 activity have different consequences for the whole-cell levels of Mps1; roscovitine can result in the near complete loss of Mps1 from the cell ***(4, 5)***, while Cdk2-specific siRNAs reduce whole-cell Mps1 levels only by roughly 25% and cyclin A-specific siRNAs have virtually no effect on whole-cell Mps1 levels ***(4)***. In addition, inhibition of the proteasome causes a 2.5-fold increase in centrosomal Mps1 levels, but causes only a modest increase in whole-cell Mps1 levels ***(4)***.

Due to unavoidable differences in sample loading such quantitative comparisons between samples require normalization to a loading control such as β-actin ***(6)***, αTubulin ***(3, 4, 7)***, or βTubulin ***(8)***. When comparing Mps1 between samples our choice has been αTubulin because its levels are not affected by Cdk2 or proteasome activity ***(3-5)***. However, Mps1 and αTubulin are present at dramatically

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different levels and using enzymatic detection methods require dramatically different film exposures (seconds for αTubulin versus minutes for Mps1) that are incompatible with detection on the same blot. This requires that the two proteins be detected on separate blots, introducing an unavoidable source of error and limiting the utility of enzymatic detection methods. In contrast, proteins of vastly different abundance can be detected simultaneously in the same lane of an immunoblot when secondary antibodies conjugated to infrared dyes are used in conjunction with the Odyssey laser scanner (LI-COR, Lincoln, NE). Unlike film detection there is no signal spread in laser scanning detection, and blots can be scanned multiple times without any loss of signal. Therefore, antigens that differ in concentration by orders of magnitude can be simultaneously measured using the same secondary antibody. Furthermore, the Odyssey can detect two differently labeled secondary antibodies at the same time, allowing four or more antigens to be measured simultaneously in the same lane of an immunoblot, provided they are well separated by size and good quality primary antibodies are available. Accordingly, based on its sensitivity and accuracy over large concentration ranges, the LI-COR Odyssey is increasingly used for detection of low abundance proteins and quantitative immunoblotting ***(3, 4, 6–8)***.

2. Materials

2.1. Cell Culture, Tansfection, and Lysis

1. Standard growth medium is Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with Penicillin/Streptomycin (Invitrogen) and 10% (v/v) Fetal Bovine Serum (Hyclone, Ogden, UT).
2. Roscovitine and MG115 (EMD Biosciences/CalBiochem, San Diego, CA) are dissolved in culture grade dimethy sulfoxide (DMSO) at 50 m*M* and 10 m*M*, respectively (unless specifically noted, compounds are from Sigma, St. Louis, MO)
3. Hydroxyurea (HU) is dissolved at 1 *M* in water (*see* **Note 1**).
4. Cdk2 and Cyclin A Smart Pool siRNAs (Dharmacon, Lafayette, CO).
5. Six well tissue culture plates (Corning, Lowell, MA).
6. Oligofectamine reagent (Invitrogen) for siRNA transfection.
7. Lysis buffer: 50 m*M* Tris-HCl pH 8.0, 150 m*M* NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.2% sodium dodecyl sulfate (SDS), 1X (v/v) mammalian protease inhibitor Cocktail (100X cocktail prepared in DMSO), and 2 m*M* 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 1 M stock prepared in water).
8. Bradford assay reagent (BioRad, Hercules, CA).

2.2. SDS-PAGE

1. 5X SDS-PAGE Sample Buffer ***(4, 5)***: 250 m*M* Tris-HCl pH 6.8, 10% (w/v) SDS, 50% glycerol, 0.5 *M* dithiothreitol, 0.5% bromophenol blue.
2. EZ-RUN Pre-Stained Protein Ladder molecular weight standard (Fisher).

2.3. Two-Color Infrared Immunoblotting

1. Transfer buffer: 25 m*M* Tris, 192 m*M* glycine, 20% (v/v) methanol.
2. Hybond Nitrocellulose membrane (GE Health/Amersham, Swampscott, NJ) (*see* **Note 2**).
3. Ponceau S reversible protein stain, premixed solution.
4. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, and 2 m*M* KH_2PO_4, pH 7.0.
5. Blocking Buffer: 3% (w/v) nonfat dry milk in PBS. Blocking buffer does not contain Tween-20 or PBS (*see* **Note 2**).
6. PBS with 0.2% Tween-20 (PBS-T).
7. Primary antibodies diluted in PBS-T: DM1A mouse anti α-Tubulin (1:10,000) (Sigma); N1 mouse anti-Mps1 (1:1,000) ***(9)*** (Invitrogen); rabbit anti-Cdk2 (1:1,000) (Sigma).
8. Secondary antibodies diluted in PBS-T: Alexa680-conjugated donkey anti-rabbit IgG (1:10,000) (Invitrogen); IRDye800-conjugated donkey anti mouse IgG (1:10,000) (Rockland Immunochemicals, Gilbertsville, PA).

3. Methods

Because different methods of inhibiting Cdk2 activity differentially effect the cytoplasmic and centrosomal pools of Mps1 ***(4)***, it is necessary to examine Mps1 levels by both immunoblot and immunofluorescence. This chapter covers the use of the LI-COR Odyssey scanner and infrared fluorescence for comparing whole cell Mps1 levels between samples. In order to compare whole-cell Mps1 levels between samples, the Mps1 signal on immunoblots from each sample must first be normalized to that of αTubulin for the same sample. Using the Odyssey scanner we can simultaneously detect both proteins in the same lane of an immunoblot.

3.1. Preparation of Samples for Immunoblotting

1. Passage and process cells according to your specific needs. For analysis of Mps1 we typically seed HeLa cells at 0.5–1×10^4 cells per well of a 6 well dish. In the specific examples used here, cells are harvested after drug treatment or siRNA transfection. For drug treatment, cells are arrested in S-phase by incubation in medium containing 4 m*M* HU for 24 hr, and then treated with 180 μM roscovitine or 5 μM MG115 for 4–24 hours in the continued presence of HU. In case of siRNA transfection, cells are transfected for 24 h, then arrested in S-phase with a 24 h HU treatment.
2. Harvest the cells according to your specific needs. Mps1 is a low abundance protein, which requires us to generate high concentration cell lysates. Accordingly, we use standard cell passaging techniques to detach cells from culture dishes, collect them by centrifugation, wash to remove media and serum, and then lyse the resulting cell pellet in 25 μl of pre-chilled lysis buffer.

3. Protein determination (optional, *see* **Note 3**): For each sample to be assayed and a blank, add to a microcentrifuge tube in order 800 µl of water, 1 µl lysate (or lysis buffer for the blank), and 200 µl undiluted assay reagent. Cap tubes, mix by inversion, and measure absorbance at 595 nm. The background corrected absorbance is then compared to a standard curve to estimate the concentration of each lysate.
4. Combine between 10 and 40 µg of protein with 5 µl of 5X sample buffer and sufficient water to bring the final volume to 25 µl in a microcentrifuge tube. Samples are heated to 95 °C for 5 min, and centrifuged at full speed for 3 min. Do not return samples to ice, because SDS from the sample buffer may precipitate.

3.2. SDS-PAGE

1. Prepare SDS-PAGE gels according to your specific needs. These instructions are given for the Hoefer SE245 Dual Gel Caster and SE260 minivertical gel electrophoresis unit, using 10 cm gel plates, 0.75 mm spacers, and 10% Anderson's modified SDS-PAGE gels ***(10)***, but are by no means specific to this system.
2. Prepare samples (**Subheading 3.1.,** step 4) while the stacking gel is polymerizing.
3. Load 7 µl of prestained protein markers in the first well (prestained markers are not boiled). Load 20 µl of the samples prepared in **Subheading 3.1, step 4** to adjacent wells.
4. Perform electrophoresis according to the requirements of your experiment. We run minigels at 20 mA constant current at room temperature.

3.3. Two-Color Immunoblotting

1. This protocol assumes the use of the BioRad Mini Transblot, but should work well with any combination of gel and transfer apparatus.
2. Perform electrophoretic transfer according to the requirements of your protocol. We perform transfers with gentle stirring at 90V for 1 h at room temperature with a Bio-Ice cooling unit present in the buffer tank to absorb the heat generated during transfer, or at 30V overnight at 4°C.
3. After transfer, remove and disassemble the cassette and stain the membrane with Ponceau S solution for 5 min at room temperature (optional). Rinse the membrane several times with water to remove background staining and assess transfer. Ponceau S, which can be recycled and reused several times, is a reversible protein stain that will dissipate in subsequent steps and does not interfere with antibody binding.
4. Block the membrane in 100 ml blocking buffer for 1 h at room temperature with gentle rocking, or at 4°C overnight. Do not use Tween or BSA in the blocking step (*see* **Note 2**).
5. If primary antibody is to be recycled, rinse the membrane twice briefly with PBS-T to remove residual blocking agent. Incubate at room temperature for 1 h with gentle rocking in enough primary antibody solution to cover the membrane without drying.
6. Remove primary antibody and wash the membrane three times for 5 min with ~50 ml PBS-T on a rocking platform. Primary antibody can often be recycled with the addition of 1 mM sodium azide (from a 1 *M* stock solution) and storage at 4°C.

7. Incubate the membrane with secondary antibody mixture in the dark at room temperature for 1 h on a rocking platform. Because the secondary antibodies are conjugated to photo-labile fluorescent dyes it is essential that this and subsequent steps be performed in the dark (*see* **Note 4**).
8. Discard the secondary antibody solution and wash the membrane with PBS-T three times for 5 min on a rocking platform. Leave the last wash in the container.
9. Rinse the glass scanning bed of the Odyssey with water and wipe clean with Kimwipes. Note that paper towels should not be used to avoid scratching the scanning bed. It is not necessary for the scanning bed to be completely dried.
10. Place the membrane on the scanning bed, protein side facing down, with one corner of the blot at the origin (in the lower left corner). The scanning bed is designed to be wet, and the presence of a small amount of PBS-T facilitates the removal of bubbles.
11. Rinse the Teflon mat with water and place it on top of the membrane. Use the Teflon roller to remove any bubbles between the scanning bed, membrane, and mat.
12. Using the Odyssey software (current version 2.1), access the scanner, enter a name for the analysis, define the area to be scanned, and set the scanning parameters of resolution, intensity (each of two channels can be set independently over two orders of magnitude), and focus offset (formats such as microtiter plates and gels that require the lasers to be focused above the scanning bed can also be analyzed on the Odyssey). As defaults we scan at 169 µM resolution, an intensity setting of 5, and a focus offset of zero.
13. Start the scanner. Once the blot has been scanned (both lasers scan the membrane simultaneously), data can be viewed as individual gray scale images, individual pseudocolored images (red for Alexa680 and green for IRDye800), or as one merged pseudocolored image. While the detection range for the Odyssey spans multiple orders of magnitude, it is possible to saturate the detectors; this is indicated by a white signal in pseudocolored images and a cyan signal in gray scale images. If a signal is saturated (most common with high abundance proteins such as the αTubulin loading control) simply reduce the intensity in that channel and scan the membrane again.
14. The signals for the protein of interest and the loading control are determined using the Odyssey software to draw boxes around each band, and must be background corrected (*see* **Note 5**). **Fig. 1A** shows S-phase arrested cells treated with DMSO, Roscovitine, or MG115, and the blot was simultaneously stained with mouse anti-Mps1 and mouse anti-αTubulin. **Fig. 1B** shows S-phase arrested cells transfected with cyclin A- or Cdk2-specific siRNAs, and the blot was simultaneously stained with three antibodies; mouse anti-αTubulin, mouse anti-Mps1, and rabbit anti-Cdk2. Note that the prestained markers, or any blue dye (including Coomassie), can be detected in the 700 channel. **Fig. 1C** and **D** show the background corrected intensity for the relevant bands from **Fig. 1A** and **B**, respectively. From these values the ratio of target protein to loading control is determined and compared between samples. Note that despite overloading of the roscovitine treated sample (**Fig. 1A** and **C**, lane 2), the normalization procedure allows its comparison to other samples; roscovitine caused a 50% decrease in Mps1 while MG115 caused a

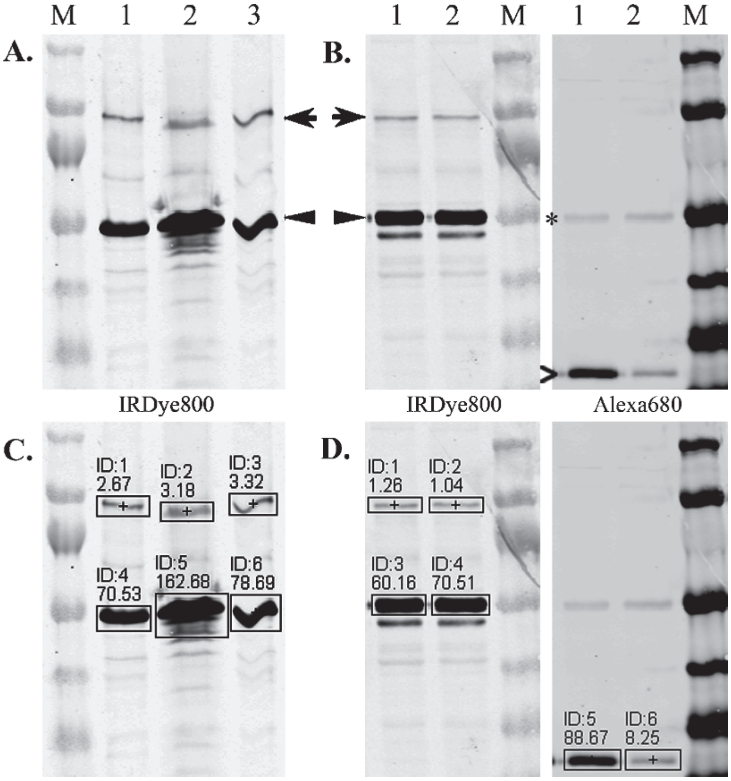
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Fig. 1. Analysis of whole-cell Mps1 levels with two-color immunoblotting. **(A)** S-phase arrested HeLa cells treated with DMSO (lane 1), roscovitine (lane 2), or MG115 (lane 3) were processed for immunoblotting with mouse anti-αTubulin and mouse anti-Mps1; M, prestained molecular weight markers. **(B)** HeLa cells transfected with cyclin A-specific (lane 1) or Cdk2-specific (lane 2) siRNAs were arrested in S-phase and processed for immunoblotting with mouse anti-αTubulin, mouse anti-Mps1, and rabbit anti-Cdk2. **(C** and **D)** Background corrected signal intensities for bands in A and B. Images show Alexa680 anti-rabbit and IRDye800 anti-mouse signals in grayscale; arrowheads, αTubulin; arrows, Mps1; caret, Cdk2; asterisk indicates cross reactivity of Alexa680 anti-rabbit with the mouse anti-αTubulin primary antibody.

10% increase, as compared to DMSO (**Fig. 1A** and **C**), and Cdk2-specific siRNA reduced Cdk2 by 93% and Mps1 by 30% compared to cyclin A-siRNA, where neither is changed ***(4)*** (**Fig. 1B** and **D**).

4. Notes

1. A highly purified source of water, either double distilled or ultra filtered, should be used for preparation of all reagents (e.g. we use water purified with a Milli-Q Biocel unit, Millipore, Billercia, MA).

2. Several factors must be taken into consideration when preparing to detect infrared fluorescent dyes. First, special membranes have been specifically designed to reduce background when using fluorescent dyes; although their routine use is not required they may reduce background when working with difficult to detect proteins and may be helpful when trouble shooting. Second, the presence of Tween-20 and other detergents in the blocking step will cause high background when scanning. However, detergent can be added once the initial blocking is complete with no ill effect. Third, the use of BSA as a blocking agent can lead to high background when scanning. Serum or nonfat dry milk is therefore recommended.
3. Because the Mps1 signal is normalized to the αTubulin loading control for each individual sample and the ratio will be the same for each sample regardless of loading, it is not strictly necessary to load an equal amount of protein for each sample. Of course loading approximately equal amounts of protein in each sample generates more presentable immunoblots.
4. After adding secondary antibody it is necessary to perform all steps in the dark. This can be accomplished in a dark room, but the need to access waste disposal makes it more practical to cover the blots and perform subsequent incubations and washes in the standard laboratory setting. This can be accomplished several ways: Light tight plastic containers; a light tight box placed on top of a rocking platform; placing containers in light-tight x-ray cassette bags; construction of light-tight bags or containers from heavy duty black plastic; or wrapping containers in foil.
5. Values measured by the Odyssey software represent the signal intensity for the respective dye and are directly proportional to the amount of secondary antibody present. However, values must be corrected for background using one of two methods. First, the software can be set to "User-defined background" to define a specific box drawn at an appropriate place on the blot as background. The software uses the average pixel intensity in this box to estimate and subtract background from the value of other boxes. Second, the software can be set to "Automatic background correction." In this mode the software uses the pixels touching the edges of each box to estimate and subtract background from that box. This is preferable because it represents a local background correction method and background may differ across the blot. The values in **Fig. 1** were determined by the later method.

Acknowledgments

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The Use of Infrared Fluorescent Dyes in Immunofluorescence Microscopy

Christopher Kasbek, Ching-Hui Yang, and Harold A. Fisk

1. Introduction

Centrosomes are microtubule organizing centers that regulate assembly of the mitotic spindle apparatus ***(1)***. Excess centrosomes such as those observed in a variety of human tumors generate aberrant mitotic spindles, and the duplication of centrosomes must be tightly controlled ***(2)***. The Mps1 protein kinase localizes to centrosomes and is required for centrosome duplication ***(3-5)***. However, Mps1 has many cellular localizations including kinetochores ***(3, 6-8)*** and nuclear pores ***(7)***, and has additional functions related to the mitotic spindle assembly checkpoint ***(9, 10)***. Recent evidence suggests that centrosome duplication specifically requires the centrosomal Mps1 pool, and that the accumulation of Mps1 at centrosomes is regulated by mutually exclusive Cdk2 phosphorylation and proteasome-mediated degradation ***(5)***. However, different means of inhibiting Cdk2 activity have different consequences for the centrosomal and whole-cell pools of Mps1 ***(5)***. Both inhibition of Cdk2 with the small molecule roscovitine and the depletion of Cdk2 activity with cyclin A-specific siRNAs leads to the loss of Mps1 from centrosomes. However, while Mps1 can be almost completely depleted from cells by roscovitine ***(3, 5)***, whole-cell Mps1 levels are virtually unchanged by cyclin A-specific siRNAs ***(5)***. In contrast, while overexpression of cyclin A only modestly increases in whole-cell Mps1 levels, it causes a 2.5-fold increase in centrosomal Mps1 levels that is sufficient to cause the over production of centrosomes during S-phase arrest ***(5)***.

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2. Materials

2.1. Cell Culture, Transfection, and Lysis

1. Standard growth medium is Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with Penicillin/Streptomycin (Invitrogen) and 10% (v/v) Fetal Bovine Serum (Hyclone, Ogden, UT).
2. Hydroxyurea (HU) is dissolved at 1 *M* in water (*see* **Note 1**).
3. Six well tissue culture plates (Corning, Lowell, MA).
4. Effectene reagents for plasmid transfection (Qiagen, Valencia, CA).

2.2. Infrared Fluorescence Microscopy

1. Pre-cleaned frosted glass microscope slides and 12 mm round glass coverslips (Fisher Scientific, Pittsburgh, PA).
2. Poly-L-lysine, sterile 0.01% solution, cell culture tested.
3. Phosphate buffered saline containing $MgCl_2$ (PBS/Mg): 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, 0.5 m*M* $MgCl_2$, pH 7.0 (*see* **Note 2**).
4. Fixative solution: 4% formaldehyde, 0.2% Triton X-100 in PBS/Mg; Prepared using 16% Electron microscopy grade formaldehyde (Ted Pella, Redding, CA), 20% Triton X-100 (Fisher), 1 *M* $MgCl_2$, and 20 X PBS.
5. Blocking buffer: 5% fetal bovine serum, 0.2% Triton X-100, 2 m*M* glycine in PBS/Mg.
6. Primary antibodies diluted in blocking buffer: Ag3 rabbit anti Mps1 (1:500) ***(4, 7)***; GTU-88 mouse anti γ-Tubulin (1:200) (Sigma).
7. Secondary antibodies diluted in blocking buffer: Alexa594-conjugated donkey anti rabbit (1:1,000) and Alexa750-conjugated donkey anti Mouse (1:200) (Invitrogen).
8. Nucleic acid stain: Hoehcst 33342, 1000 X stock solution at 1 mg/ml in water.
9. Humidified chamber. Place a wet paper towel in the bottom of an empty pipette tip box. For incubation in the dark, it is typically sufficient to place this humidified chamber in a drawer. Once the lid is closed the chamber will be sufficiently humidified to prevent the evaporation of small volumes of antibody.
10. SlowFade Mounting Medium (Invitrogen).
11. Clear nail polish

3. Methods

Because different methods of inhibiting Cdk2 activity differentially effect the cytoplasmic and centrosomal pools of Mps1 ***(5)***, it is necessary to examine Mps1 levels by both immunoblot and immunofluorescence. This chapter describes the use of infrared fluorescence microscopy for the analysis of the centrosomal Mps1 pool. Typically, we analyze whole cell and centrosomal Mps1 levels in identical populations by placing glass coverslips into the wells of a six well plate. After appropriate experimental manipulation, coverslips are harvested for Indirect Immunofluorescence (IIF), and the cells remaining on the surface of the dish are harvested for immunoblot analysis.

The comparison of centrosomal Mps1 levels requires that cells are processed and imaged under identical conditions. Practically, this limits such analysis to overexpression techniques where adjacent untransfected cells and transfected cells on the same coverslip can be imaged concurrently. The use of infrared fluorescent secondary antibodies and an efficient infrared light source and filter set allows centrosomes to be identified using infrared wavelengths, reserving the visible channels for imaging DNA, GFP, and Mps1.

3.1. Preparation of Samples for Infrared Fluorescence Microscopy

1. For microscopy cells are grown on acid washed and poly-L-lysine coated 12 mm round glass coverslips (*see* **Note 3**) added directly to 6-well plates. Coverslips are typically coated with poly-L-lysine (*see* **Note 4**) before plating cells.
2. Passage and process cells according to your specific needs. For analysis of Mps1 we typically seed HeLa cells at 1×10^4 cells per well of a 6 well dish. In the specific examples used here, coverslips are harvested after a 24 h plasmid transfection (in the example here cells are transfected with the pECE-GFP cyclin A ***(5)*** expression plasmid) followed by a 24 h treatment with 4 mM HU to achieve S-phase arrest.

3.2. Infrared Fluorescence Microscopy

1. Transfer individual coverslips from six-well plates into a 24-well plate, maintaining the same orientation as in the culture dish so that cells continue to face the ceiling.
2. Fix cells as required for the specific antigens and antibodies to be used, and rehydrate if necessary. We typically visualize centrosomes in cells that have been fixed for ten min at room temperature in 4% formaldehyde and 0.2 % triton X-100 in PBS/Mg, and remove excess fixative by washing four times with ~0.5 ml PBS containing 0.5 m*M* $MgCl_2$ (PBS/Mg) (*see* **Note 2**). Formaldehyde is volatile, toxic, and a potential carcinogen. Formaldehyde fixation should be performed in a fume hood, and both used fixative and the first wash should be disposed of as hazardous waste.
3. Incubate coverslips in blocking buffer for 1 h at room temperature, or overnight at 4°C, in a dark humidified chamber.
4. Incubate coverslips in primary antibody solution at room temperature for 1 h in a dark humidified chamber. Primary antibody incubations can be performed concurrently or consecutively according to the need to recycle antibodies.
5. Wash coverslips four times with ~0.5 ml PBS/Mg.
6. Incubate coverslips in secondary antibody solution at room temperature for 1 h in a dark humidified chamber, then wash coverslips four times with ~0.5 ml PBS/Mg. Do not remove the final wash. Secondary antibodies are typically not recycled and can be incubated concurrently with Hoechst.
7. Invert coverslips onto a small drop (~5 µl) of mounting medium. Taking care not to move coverslips, remove residual mounting medium and clean coverslips by gentle blotting with a damp paper towel. Once coverslips are dry seal around the edges with nail polish. By staggering, up to eight coverslips can fit on a single microscope slide.

8. Cells are then imaged under a 63x oil immersion objective using a Xenon lamp (*see* **Note 5).** Hoechst is visualized through a standard DAPI filter set, GFP is visualized through a narrow band FITC filter set, Alexa 594 is visualized through a standard Texas Red filter set, and Alexa 750 is visualized through a Cy7 filter set.
9. Cells are identified using Hoechst, and transfected cells are distinguished by their GFP signal. The centrosomal protein γTubulin is visualized in the infrared channel (*see* **Note 5**) and is used to identify the centrosomal pool of Mps1. **Fig 1** shows typical results from the overexpression of GFP-cyclin A in HeLa cells. The Hoechst signal **(Fig. 1A)** reveals two cells in the field, the GFP signal **(Fig. 1B)** identifies a cell overexpressing GFP-cyclin A, the Alexa 594 signal reveals the Mps1 distribution **(Fig. 1C),** and the Alexa 750 signal identifies the location of centrosomes **(Fig. 1D).** The insets in **Fig. 1E** show 4-fold magnified images of the GFP, γTubulin, and Mps1 signals from the region surrounding a centrosome in the nontransfected cell, and that in **Fig. 1F** for the GFP-cyclin A-transfected cell. The increased Mps1 signal in the GFP-cyclin A expressing cell is evident, and was measured at 2.5-fold ***(5)*** using a local background correction method developed by Howell et al. ***(11)***.

4. Notes

1. A highly purified source of water, either double distilled or ultrafiltered, should be used for preparation of all reagents (e.g. we use water purified with a Milli-Q Biocel unit, Millipore, Billercia, MA).
2. Because divalent cations inhibit the action of trypsin-like enzymes and are required for the function of cell adhesion molecules, Calcium and Magnesium-free PBS is used when passaging cells. However, the inclusion of Magnesium and/or Calcium can prevent the loss of cells from coverslips during IIF (at 0.5 m*M* we have found no difference between $MgCl_2$ and $CalCl_2$).
3. To acid wash coverslips, gently place a box of 12 mm round glass coverslips (approximately a gross) into the bottom of a glass beaker, cover with 1 N HCl and incubate at 50–60 °C overnight. After extensively washing the coverslips with water, they are equilibrated to 70% ethanol with two brief 70% ethanol rinses and incubated in 70% ethanol for one h at room temperature. After this sterilization, coverslips are equilibrated to 95% ethanol with two brief 95% ethanol rinses, placed individually onto clean, sterile sheets of 3MM chromatography paper (approximately 100 coverslips per sheet is practical), then dried in a tissue culture hood under a UV lamp. Once dry, coverslips can be stored in bulk in a sterile 100 mm tissue culture dish.
4. For each coverslip to be coated with poly-L-lysine place a small drop (≤1 μl) of water or PBS on the surface of the dish and place a single coverslip directly on each drop. The surface tension created by the liquid will

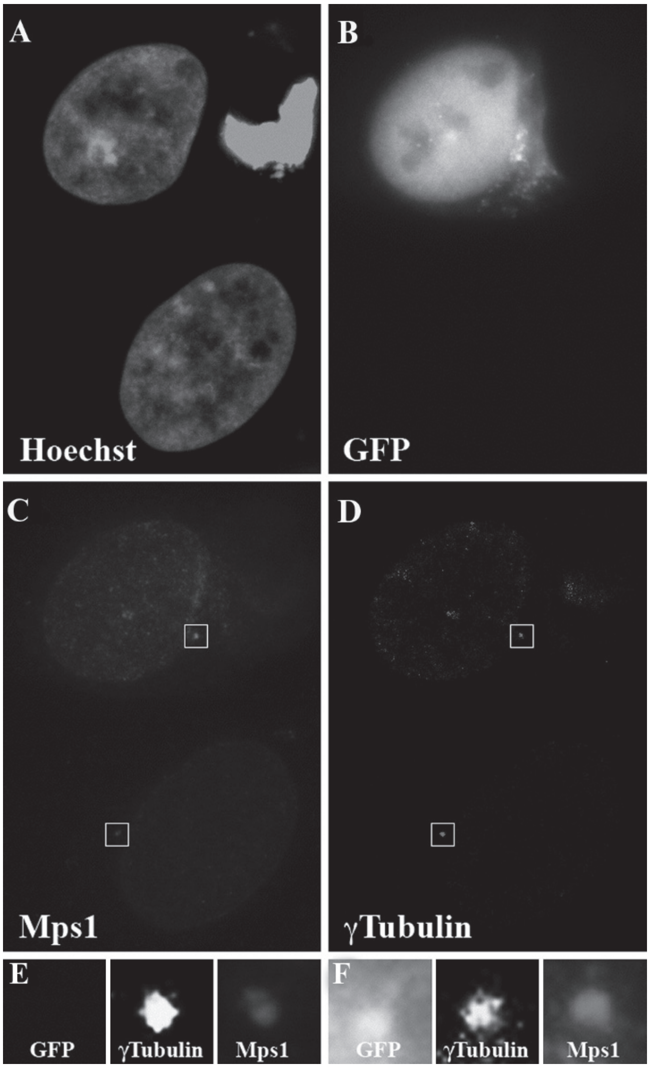

Fig 1. Identification of the centrosomal Mps1 pool using infrared fluorescence. **(A-F)** HeLa cells expressing GFP-cyclin A were arrested in S-phase and analyzed by IIF with antibodies against Mps1 and the centrosomal protein γTubulin. **(A)** Hoechst staining reveals two cells. **(B)** The GFP signal identifies the cell expressing GFP-cyclin A. **(C)** Alexa594 anti-rabbit reveals the Mps1 distribution. **(D)** Alexa750 anti-mouse identifies the position of centrosomes. Boxes in C and D indicate the region surrounding a centrosome in each cell. **(E and F)** Digitally magnified images showing the GFP, γTubulin, and Mps1 signals in the boxes surrounding the centrosomes in C and D as indicated. **(E)** A centrosome from the untransfected cell. **(F)** A centrosome from the GFP-Cyclin A expressing cell.

prevent the coverslips from moving during coating. Space the drops so that coverslips do not touch each other or the side of the dish. Pipette 50–100 µl of poly-L-lysine solution onto each coverslip. Discard coverslips that repel the poly lysine solution. Incubate for 1 h in a tissue culture hood. Remove liquid by placing an aspirator at the edge of the coverslip, and air-dry coverslips by drawing air across the surface. When coating multiple coverslips remove the majority of liquid from all coverslips, and then air-dry coverslips one by one. Wash coverslips four times with 5 ml of PBS per well for a 6-well plate, leaving the final wash until just before cells are plated. Coverslips can also be coated in bulk (e.g. in a 100 mm dish) and transferred to the experimental plate.

5. Visualization of Alexa750 and other infrared dyes requires the appropriate light source, filter set, and camera. We use a Xenon lamp, which illuminates more efficiently in the infrared wavelengths than Mercury lamps. Appropriate excitation and emission filter sets must also be used. We use the Olympus Cy7 filter set. Most digital microscope cameras can detect infrared fluorescence to some degree, although many cameras have infrared shields that must be removed. However, detection is optimal with cameras such as the QCAM Retiga Exi FAST 1394 with expanded sensitivity in the infrared wavelengths. IRDye800 can also be used in microscopy, but filter sets are not commercially available and must be special ordered (see the LI-COR website at http://www.licor.com/bio/IRDyes/IRReag.jsp for more information). Infrared fluorescence is not visible by eye, and the use of Alexa750 or IRDye800 for visualization requires a digital imaging software package capable of real time imaging (we use Slidebook 4.0 from Intelligent Imaging Innovations, Denver, CO)

Acknowledgments

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PART IV

CHEMICAL MODIFICATION OF PROTEINS AND PEPTIDE PRODUCTION AND PURIFICATION

82

Carboxymethylation of Cysteine Using Iodoacetamide/Iodoacetic Acid

Alastair Aitken and Michèle Learmonth

1. Introduction

If cysteine or cystine is identified in a protein it requires modification in order to be quantified. Thiol groups may be blocked by a variety of reagents including iodoacetic acid and iodoacetamide. Iodoacetate produces the *S*-carboxymethyl derivative of cysteine, effectively introducing new negative charges into the protein. Where such a charge difference is undesirable, iodoacetamide may be used to derivatize cysteine to *S*-carboxyamidomethylcysteine (on acid hydrolysis, as for amino acid analysis, this yields *S*-carboxymethylcysteine). The charge difference between these two derivatives has been utilized in a method to quantify the number of cysteine residues in a protein (***[1]***, *see* Chapter 115).

Carboxymethylation may be carried out without prior reduction to modify only those cysteine residues that are not involved in disulfide bridges.

If the protein is to be analyzed using gas-phase protein microsequencing, the derivatizing agent of choice is commonly vinylpyridine as this produces a well separated phenylthiohydantoin (PTH) derivative (PTH-*S*-pyridylethylcysteine), *see* Chapter 85.

2. Materials

1. Denaturing buffer: 6 *M* guanidinium hydrochloride (or 8 *M* deionized urea, *see* **Note 1**) in 0.6 *M* Tris-HCl, pH 8.6.
2. 4 m*M* Dithiothreitol (DTT), freshly prepared in distilled water.
3. β-Mercaptoethanol.
4. Sodium dodecyl sulfate (SDS).
5. Oxygen-free nitrogen source.
6. 500 m*M* Iodoacetic acid in distilled water, pH adjusted to 8.5 with NaOH. This is light sensitive and although the solution may be stored in the dark at −20°C, it is better to use it freshly prepared.
7. 500 m*M* Iodoacetamide freshly prepared in distilled water.

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8. 5 m*M* and 50 m*M* Ammonium bicarbonate in distilled water.
9. 0.1% (v/v) Trifluoracetic acid (TFA), high-performance liquid chromatography (HPLC) grade.
10. Acetonitrile, far UV HPLC grade (e.g. Romil, Cambridge, UK).
11. Microdialysis kit (such as that supplied by supplied by Pierce-Warriner).
12. HPLC apparatus.

3. Method

3.1. Large-scale Reaction

1. Dissolve protein (up to 30 mg) in 3 mL of 6 *M* guanidium hydrochoride (or 8 *M* deionized urea), 0.6 *M* Tris-HCl, pH 8.6.
2. Add 30 µL of β-mercaptoethanol (or 5 µL 4 *M* DTT) and incubate under N_2 for 3 h at room temperature. Some proteins require the presence of 1% SDS with an incubation time of up to 18 h at 30–40°C.
3. Add 0.3 mL of colorless iodoacetate (*see* **Note 2**) or iodoacetamide, with stirring, and incubate in the dark for 30 min at 37°C.

3.2 Small-scale Reaction

1. Dissolve the protein (1–10 µg) in 50 µL of denaturing buffer in an Eppendorf tube and flush with N_2.
2. Add an equal volume of 4 m*M* DTT solution to give a final concentration of 2 m*M*.
3. Wrap the tube in aluminium foil and then add 40 mL of iodoacetic acid (or iodoacetamide) dropwise with stirring. Keep under N_2, and incubate in the dark for 30 min at 37°C (*see* **Note 3**). Proceed to **Subheading 3.3.**

3.3. Purification

The removal of excess reagents may be carried out in a variety of ways.

1. Desalting on a 20 × 1 cm Sephadex G10 or Biogel P10 column, eluting with 5 m*M* ammonium bicarbonate, pH 7.8.
2. Dialysis or microdialysis using 50 m*M* ammonium bicarbonate, pH 7.8, as dialysis buffer.
3. By HPLC using a C_4 or C_8 matrix such as an Aquapore RP-300 column, equilibrated with 0.1% (v/v) TFA and eluting with an acetonitrile –0.1% TFA gradient (*see* **Note 4**).

4. Notes

1. Deionization of urea: Urea should be deionized immediately before use to remove cyanates, which react with amino and thiol groups. The method is to filter the urea solution through a mixed bed Dowex or Amberlite resin in a filter flask.
2. The iodoacetic acid used must be colorless. A yellow color indicates the presence of iodine, which will rapidly oxidize thiol groups, preventing

alkylation and may also modify tyrosine residues. It is possible to recrystallize from hexane.

3. Reductive alkylation may also be carried out using iodo–[^{14}C]acetic acid. The radiolabeled material should be diluted to the desired specific activity before use with carrier iodoacetic acid to ensure an excess over total thiol groups.
4. If the HPLC separation is combined with mass spectrometric characterization, this level of TFA (which is required to produce sharp peaks and good resolution of peptide) results in almost or complete suppression of signal. In this case it is recommended to use the new "low TFA," 218MS54, reverse-phase HPLC columns from Vydac (300 Å pore size). They are available in C_4 and two forms of C_{18} chemistries and in 1 mm diameter columns that can be used with as little as 0.005% TFA without major loss of resolution and minimal signal loss.

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83

Performic Acid Oxidation

Alastair Aitken and Michèle Learmonth

1. Introduction

Where it is necessary to carry out quantitative amino acid analysis of cysteine or cystine residues, carboxymethylation (Chapter 82) or pyridethylation (Chapter 85) is not the method of choice as it may be difficult to assess the completeness of the reactions. It is often preferable to oxidize the thiol or disulfide groups with performic acid to give cysteic acid. This also has the effect of converting methionine residues to methionine sulfone. Tyrosine residues may be protected from reaction by the use of phenol in the reaction mixture.

Performic acid oxidation is also used in the determination of disulfide linkages by diagonal electrophoresis (*see* Chapter 113).

2. Materials

1. 30% (v/v) Hydrogen peroxide. **Note**: Strong oxidizing agent.
2. 88% (v/v) Formic acid.
3. Phenol (CARE). **Note:** Toxic and corrosive.
4. 1 m*M* Mercaptoethanol.

3. Method

1. Add 0.5 mL of 30% (v/v) H_2O_2 to 4.5 mL of 88% formic acid containing 25 mg of phenol.
2. After at least 30 min at room temperature, cool to 0°C.
3. Add this reagent (performic acid, HCOOOH) to the protein sample at 0°C to result in a final protein concentration of 1%.
4. After 16–18 h, dilute the solution with an equal volume of water and dialyze against two changes of 100 volumes of water at 4°C, and finally dialyze against 100 volumes of 1 m*M* mercaptoethanol at 4°C.

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5. The derivatized protein can then be lyophilized or dried in a hydrolysis tube ready for acid cleavage and subsequent amino acid analysis.

Elution positions for separations of derivatized amino acids by ion-exchange chromatography are shown in **Table 1**.

Table 1
Elution Order of Amino Acids and Derivatives from Ion-Exchange Amino Acid Analyzers

1. O-Phosphoserine
2. O-Phosphothreonine
3. Cysteic acid
4. Urea
5. Glucosaminic acid
6. Methionine sulphoxides
7. Hydroxyproline
8. CM-Cysteine
9. Aspartic acid
10. Methionine sulphone
11. α-Methyl aspartic acid
12. Threonine
13. Serine
14. Asparagine
15. Glutamine
16. α-Methyl serine
17. Homoserine
18. Glutamic acid
19. α-Methyl glutamic acid
20. Proline
21. S-methylcysteine
22. Glycine
23. Alanine
24. S-Ethylcysteine
25. Glucosamine
26. Mannosamine
27. Galactosamine
28. Valine
29. Cysteine
30. Methionine
31. α-Methylmethionine
32. Isoleucine
33. Leucine
34. Norleucine

(continued)

Table 1
(Continued)

35. Tyrosine
36. Phenylalanine
37. Ammonia
38. Hydroxylysine
39. Lysine
40. 1-Methylhistidine
41. Histidine
42. 3-Methylhistidine
43. Tryptophan
44. Pyridylethyl-cysteine
45. Homocysteine thiolactone
46. Arginine

The precise elution order may vary depending on the exact instrument and the precise temperature, molarity, and pH of buffers.

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Succinylation of Proteins

Alastair Aitken and Michèle Learmonth

1. Introduction

Modification of lysine with dicarboxylic anhydrides such as succinic anhydride prevents the subsequent cleavage of lysyl peptide bonds with trypsin. In addition, modified proteins act as better substrates for proteases. Succinic anhydride reacts with the ε-amino group of lysine and the amino-N-terminal α-amino group of proteins, in their non-protonated forms, converting them from basic to acidic groups ***(1)***. Thus one effect of succinylation is to alter the net charge of the protein by up to two charge units. This is an effect that has been exploited for the counting of integral numbers of lysine residues within a protein ***(2)***.

Succinic anhydride has been reported to react with sulfydryl groups. It is therefore advisable to modify any cysteines within the protein (*see* Chapters 82 and 85) prior to succinylation.

The reaction occurs between pH 7.0 and 9.0. The reaction is carried out using an approx 50-fold excess of the anhydride over native or carboxymethylated protein.

2. Materials

1. 8 *M* deionized urea (*see* **Note 1**).
2. Distilled water containing up to 0.1 *M* NaCl or 0.2 *M* sodium borate buffer, pH 8.5.
3. Succinic anhydride.

3. Method

1. The protein (5 mg) should be dissolved in 5 mL of buffer (*see* **Note 2**).
2. A pH electrode should be placed within the solution to allow monitoring of the pH. The solution should be continuously stirred.

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3. The solid succinic anhydride should be added in 0.5 mg portions over a period of 15 min to 1 h to give a 50-fold excess. The pH should be adjusted back to 7 with sodium hydroxide after each addition.
4. The reaction should be allowed to proceed for at least 30 min after the last addition.
5. The modified protein may be separated from the side products of the reaction by dialysis against 50 m*M* ammonium carbonate buffer or by gel filtration (e.g., Sephadex G25) (*see* **Note 3**).

4. Notes

1. Deionization of urea: Urea should be deionized immediately before use to remove cyanates, which react with amino and thiol groups. The method is to filter the urea solution through a mixed-bed Dowex or Amberlite resin in a filter flask.
2. The choice of buffer is dependent on the solubility of the protein. Distilled water may be used or it may be necessary to use 0.1 *M* NaCl or 0.2 *M* sodium borate buffer in the presence of 8 *M* deionized urea.
3. Other dicarboxylic anhydrides may be used. Particularly common is citraconic anhydride ***(3)***. This reagent has the advantage of reversibility, being readily removed with acid. When used in conjunction with trypsin, the proteolytic reaction may be stopped by reducing the pH, which will also remove the lysine blocking groups.

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85

Pyridylethylation of Cysteine Residues

Malcolm Ward

1. Introduction

To help maintain their three-dimensional structure, many proteins contain disulfide bridges between cysteine residues. Cysteine residues can cause problems during Edman sequence analysis, and quantification of cysteine and cystine by amino acid analysis is difficult since these residues are unstable during acid hydrolysis.

Chemical modification of cysteine residues can enhance the solubility of the protein, enable more effective enzymatic digestion with proteases, such as trypsin, and facilitate quantification by amino acid analysis.

Oxidation with performic acid can be used to convert cysteine and cystine to cysteic acid ***(1)*** (and *see* Chapter 83). This, however, can lead to other nondesirable side reactions, such as oxidation of methionine residues and destruction of tryptophan residues. Alkylation with iodoacetic acid has been used extensively, since this enables the addition of negative charges to the protein. The use of iodoacetic acid containing ^{14}C provides a means of incorporating a radioactive label into the polypeptide chain (*see* Chapter 82) ***(2)***.

The method described in this chapter is an effective alternative, where reduction and alkylation can be achieved in one step. 4-Vinylpyridine is used to convert cysteine residues to *S*-pyridylethyl derivatives. The *S*-pyridylethyl group is a strong chromophore at 254 nm, which facilitates the detection of cysteine containing peptides as well as aiding the identification of cysteine residues during Edman sequencing (*see* **Note 1**).

2. Materials (*see* Note 2)

1. Denaturing buffer: 0.1 *M* Tris-HCl, pH 8.5, 6 *M* guanidine hydrochloride.
2. 4-Vinylpyridine: Store at −20°C.
3. 2-Mercaptoethanol.

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3. Method

1. Dissolve 10–50 µg protein/peptide in the denaturing buffer (1 mL).
2. Add 2-mercaptoethanol (5 µL) and 4-vinyl-pyridine (2 µL) to the sample tube and shake. Since both reagents are extremely volatile and toxic, all experimental work should be carried out in a fumehood wearing appropriate safety clothing.
3. Blow nitrogen gas over the reaction mixture to expel any oxygen.
4. Seal the tubes with a screw cap.
5. Allow the reaction to proceed at 37°C for 30 min.
6. After this time, an aliquot of the reaction mixture may be taken and analyzed by mass spectrometry. Bovine insulin is shown as an example. This protein contains two interchain bridges and one intrachain bridge (**Fig. 1**). The matrix-assisted laser desorption ionization (MALDI) mass spectrum of reduced and alkylated insulin shows molecular ions at m/z 2763 and m/z 3612 corresponding to the two fully alkylated peptide chains (**Fig. 2**) (*see* **Note 3**). There are no other signals present, indicating that no side reactions have occurred.
7. The sample can now be loaded onto a hydrophobic column for either N-terminal sequence analysis, using the HPG1000A protein sequencer, or *in situ* enzymatic or chemical digestion.

4. Notes

1. The PTH-pyridylethylcysteine derivative can be readily assigned during Edman sequence analysis. The relative elution position on the HPLC system of the Applied Biosystems gas-phase sequencer is between PTH-valine and diphenylthiourea. On the Hewlett Packard G1000A system, PTH-pyridylethylcysteine elutes at 14.2 min just before PTH-methionine.
2. All reagents should be of the highest quality available.
3. The pyridylethylation reaction as described allows for fast, effective derivatization of cysteine residues. The reaction is efficient, giving a high yield of fully alkylated product with no side products.
4. The concept of monitoring chemical reactions by mass spectrometry is not new, yet the sensitivity and speed of MALDI provide a means of examining reaction products to enable the controlled use of reagents that have previously proven troublesome. A recent publication by Vestling et al. *(3)* describes the controlled use of BNPS Skatole, a reagent that to date has

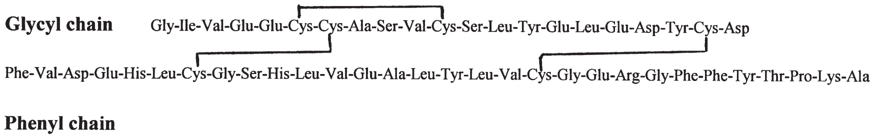
Glycyl chain
Gly-Ile-Val-Glu-Glu-Cys-Cys-Ala-Ser-Val-Cys-Ser-Leu-Tyr-Glu-Leu-Glu-Asp-Tyr-Cys-Asp
Phe-Val-Asp-Glu-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala
Phenyl chain

Fig. 1. Amino acid sequence of insulin showing position of disulfide bonds.

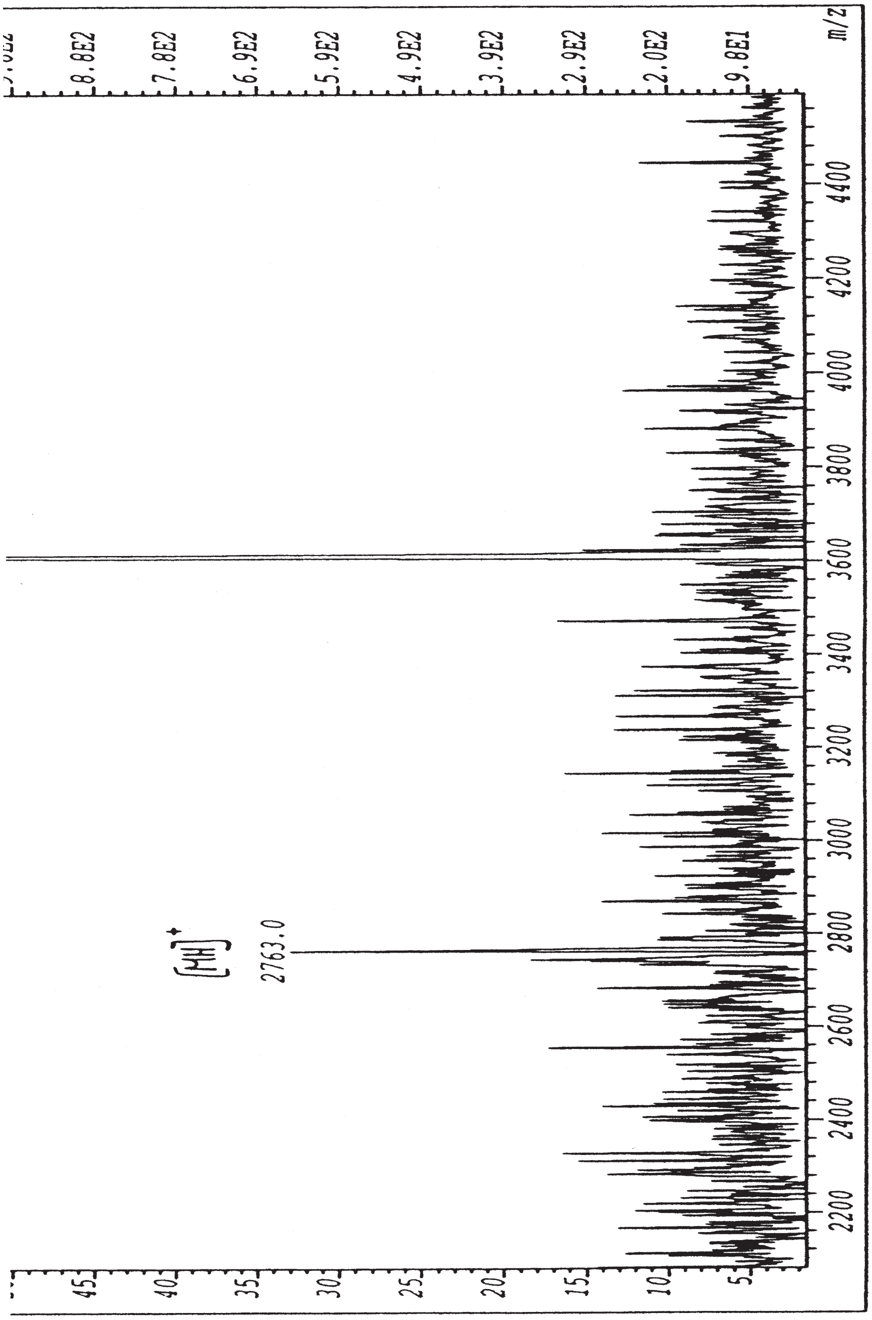

Fig. 2. The MALDI mass spectrum of reduced and alkylated insulin.

been seldom used owing to side reactions. The reactivity of such a reagent may be more widely used in the future now that the progress of the reaction can be easily monitored.

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86

Side Chain Selective Chemical Modifications of Proteins

Dan S. Tawfik

1. Introduction

Chemical modifications of proteins may be performed simply and rapidly to provide preliminary data regarding the role of particular amino acids in a given protein. Many reviews and books cover these aspects of protein chemistry; only a few are cited here ***(1–4)***. In particular, the book by Means and Feeney ***(1)***, although about 30 yr old, is an excellent introduction and a practical guide to this field. Notably, even in the era of molecular biology, when site-directed mutagenesis has become widely accessible (including with nonnatural amino acids), selective chemical modifications are still applied regularly. In most cases, chemical modifications are used, often together with site-directed mutagenesis, to either identify or confirm the role of active site residues (for recent examples *see* **ref.** ***5–9***). But chemical modifications are also applied for the generation of improved and modified proteins for a variety of applications ***(10–12)***. More recent applications in the field of chemical biology include proteomics (e.g. protein arrays and mass spectroscopy analyses; **ref. 13**), and a broad range of side-chain modifications, including groups that mimic posttranslational modifications ***(14–16)***, and selective chemical modifications of proteins that make use of specific enzymes ***(17,18)***.

The modifications discussed in the following chapters are side chain selective, that is, *under appropriate conditions*, the reagents mentioned in this chapter (and additional reagents mentioned in **refs.** ***1–4***) react specifically with a single type of amino acid side chain. Hence, loss of activity (enzymatic, binding, or other biological activity) following treatment of the protein with such a

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modifying reagent is considered to be an indication for the role of that side chain in the active site of the protein.

Data obtained by side chain modifications must be analyzed with caution (as is the case for data obtained by genetic site-directed mutagenesis). Loss of activity on treatment with a reagent might be the result of conformational changes or other changes that occur far from the active site. Some of the reagents, in particular when applied in large excess or under inappropriate conditions, may react with more than one type of side chain or may even disrupt the overall fold of the protein. In general, the type of modifications that alter the size of a particular residue, but not its charge, are preferred (*see* Chapters 90 and 91). In addition, the reactivity of a certain type of side chain in a protein varies by several orders of magnitude owing to interactions with neighboring groups that affect the accessibility and reactivity. For example, the pK_a of the carboxylate side chain of aspartic acid is generally approx 4.5; however, interactions with other side chains may increase the pK_a by more than three units. This change will have a major effect on the reactivity of such a carboxylate group toward the modifying reagent. Dramatic pK_a changes are often found in active sites; thus, certain residues might be particularly difficult to modify, thereby forcing conditions that result in a nonspecific loss of activity.

A number of quite simple experiments may strongly support the results obtained by chemical modifications:

1. A simple control that allows the modification to be ascribed to the active site of a protein is to demonstrate protection (i.e. lack of modification) in the presence of a specific ligand to that site, for example, a hapten for an antibody, a substrate or an inhibitor for an enzyme (for an example *see* Chapter 87).
2. The extent of modification is determined primarily by the molar excess of the modifying reagent, but also by other conditions such as pH, temperature, and reaction time. Reliable and reproducible results are generally obtained only after a wide range of reagent concentrations are applied under different reaction conditions. Following these modifications, one should determine not only the remaining biological activity but also, when possible, the number of modified side chains (details for each reaction are provided in the following chapters). These data may allow one to assess to what extent the modification is indeed site specific (e.g., loss of activity is the result of modification of one or two amino acids of the type modified) or whether loss of activity is due to a complete disruption of the protein due to, for example, the modification of a large number of amino acids.
3. Some of the modifications are reversible; for example, histidine side chains modified by diethyl pyrocarbonate can be recovered by a short treatment with hydroxylamine. Recovery of the activity of the modified protein following this treatment may demonstrate the specificity of the modification (*see* Chapter 88). Additional reversible modifications are described in Chapters 91 and 93.
4. Demonstrating pseudo-first-order kinetics for the inactivation of the protein may indicate that the modification proceeds like an ordinary bimolecular reaction and not via the formation of a binding complex as with affinity labelers or suicide inhibitors.

In the following chapters, I have provided basic protocols for the specific modifications of different side chains; these, or very similar, protocols can be applied with other reagents as well (*see* **refs. *1–4***). The first protocol for the nitration of tyrosine side chains with tetranitromethane (Chapter 87) is written for a specific protein (an antidinitrophenyl [DNP] antibody) and provides as many experimental details as possible. Hence it is recommended to read Chapter 87 (including the Notes) before applying any of the other modifications described in Chapters 88 to 93.

1.1. General Notes

1. To avoid misleading results it is important to be familiar with the *chemistry* of each of the reagents (*see* **refs. *1–4*** and references therein). A detailed mechanistic discussion is beyond the scope of this book, although examples for typical problems or side reactions are given in each chapter.
2. Many of the reagents and solvents described are harmful and should be used only in a well-ventilated hood and while applying other precautions such as wearing suitable gloves.
3. Examine the buffer you intend to use with your protein in light of the modification reaction. For example, while amidating carboxylate groups (*see* Chapter 90), the use of an acetate buffer, or of any other buffer that contains carboxylate groups or other nucleophiles (e.g., Tris buffer), should obviously be avoided. Likewise, the use of certain organic solvents should be avoided (e.g., acetone with 2-hydroxy-5-nitrobenzylbromide, *see* Chapter 92).
4. Most of the reagents described in the following chapters are reactive with water; however, in some cases, quenching the excess of unreacted reagent is required (for an example *see* Chapter 87).
5. Chemically modified proteins are often unstable. Likewise, some of the modifications are removed even under mild conditions. This must be taken into consideration while the protein is purified and its activity is being determined. Hence, when possible, it is best to determine the results of the modification reaction (i.e., the remaining biological activity and the number of modified residues) immediately after the reaction and minimize further manipulations of the protein (e.g., dialysis or gel filtration).

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Nitration of Tyrosines

Dan S. Tawfik

1. Introduction

Tetranitromethane (TNM) reacts with the phenolic side chain of tyrosine under relatively mild conditions to give 3-nitrotyrosine ***(1)***. The protocol described in this chapter was developed for an anti-DNP antibody, but can be used with any other antibody or protein. The major side reaction is oxidation of thiols although under more extreme conditions tryptophans and methionines might be oxidized as well. As the reactive species is the phenolate ion, the main factor controlling the reactivity of protein tyrosyl side chains toward nitration by TNM is pH. Increasing the pH will usually enhance the rate of modification.

The number of nitrated tyrosines can be determined spectrophotometrically during the reaction. In addition, the stability of nitrotyrosine allows the specific site of modification to be determined by amino acid analysis of cleaved fragments of the protein. Finally, 3-nitrotyrosine has a much lower pK_a than tyrosine. Thus, examining the pH activity profile of the nitrated protein, for example, the binding of the protein to its ligand can be readily exploited not only to demonstrate the specificity of the modification but also to engineer the binding properties of the protein (*see* **Subheading 3.2.** and **refs. *2–5***).

2. Materials

1. Tris-buffered saline (TBS) 8.0: 0.05 *M* Tris-HCl, pH 8.0, 0.15 *M* NaCl.
2. 1–2 mg/mL monoclonal or polyclonal antibody preparation (purified by protein A affinity chromatography).
3. Acetonitrile.
4. TNM. Store in aliquots at −20°C.

   Note: TNM should be handled with care. Preparation of aliquots and of stock solutions should be done in a ventilated hood and with suitable gloves!

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5. 2-Mercaptoethanol.
6. Phosphate buffered saline (PBS): 0.01 *M* phosphate buffer, pH 7.4, 0.15 *M* NaCl.
7. Dialysis tubes: 10,000 molecular weight cutoff.

3. Method

3.1. Nitration of Tyrosines with Tetranitromethane

1. Dialyze the antibody against TBS 8.0 (4 h at 4°C). Determine the protein concentration in the sample by measuring the absorbance at 280 nm (for IgG, $\varepsilon = 1.45\,\text{cm}^{-1}\,\text{mg}^{-1}\,\text{mL}$), and adjust it with TBS 8.0 to optical density (OD) 1.09 or 0.75 mg/mL (= 5 µ*M* antibody = 10 m*M* sites).
2. Prepare a set of TNM solutions in acetonitrile: 0, 2.1, 10.5, 42, 105, and 420 m*M* (corresponding to 21 times the final reagent concentration or to 0, 10, 50, 200, 500 and 2000 molar ratios of TNM per antibody sites) (*see* **Note 1**).
3. Add 5 µL of each of the TNM solutions to 100 µL aliquots of the cold antibody solution in Eppendorf tubes immersed in an ice bath. Incubate, with occasional stirring, for 1.5 h at 4°C and then for 30 min at room temperature.
4. Quench the reaction by adding 2 µL of 2-mercaptoethanol, to the samples containing 0–200 fold excess of TNM, and 10 µL to the samples containing 500–2000 fold excess of TNM. Incubate for 15 min at room temperature.
5. Dilute the samples with TBS to a total volume of 500 µL and dialyze them against TBS or PBS (at least twice; each round for 4 h at 4°C).
6. Determine the activity of the various antibody samples by enzyme-linked immunosorbent assay (ELISA) (or any other immunoassay) at increasing antibody dilutions (e.g., 1:50 up to 1:50,000 in PBS).
7. Determine the number of 3-nitrotyrosines per antibody molecule by measuring the optical density at 428 nm ($\varepsilon = 4100\,M^{-1}\,\text{cm}^{-1}$ at pH ≥ 8.5; *see* **Note 2**).
8. For modification in the presence of the hapten-dinitrophenol (DNPOH): Incubate the antibody with 1 m*M* DNPOH for 30–60 min at 4°C.
9. Proceed with the addition of teranitromethane, quenching and dialysis as described in the preceding (*see* **Note 3**).
10. Determine the number of 3-nitrotyrosines by measuring the optical density at 428 nm (and compare it to the number of tyrosines modified under the same conditions in the absence of the hapten) (*see* **Note 4**).

3.2. pH Dependency of Binding of Nitrated Antibodies

Nitration of the phenolic group of tyrosine induces a dramatic shift in the pK_a of this residue. The pK_a of tyrosine is normally around 10; thus the hydroxyl group is mostly protonated under pH ≤ 9.5. In contrast, the pK_a of 3-nitrotyrosine is around 7.0 *(1)*; hence, loss of activity of the nitrated protein is often the result of deprotonation of the hydroxyl group at pH above 7.0. In such cases, activity could be recovered at pH < 6.0, at which the hydroxyl of the nitrated tyrosine regains its proton. This was originally demonstrated with several antibodies (including an anti-DNP antibody) in which a pH dependency of binding was observed after site-specific modification of tyrosine with TNM ***(2)***. More recently, this approach was applied to other antibodies ***(3)***, and also with avidin, leading to pH-dependent, reversible biotin binding ***(4)***. Recovery and loss of binding of these antibodies to the corresponding haptens (at pH < 6.0 and at pH > 8.0, respectively) were ascribed to the protonation and deprotonation of the hydroxyl group of a 3-nitrotyrosine side chain at their binding sites.

This approach can be utilized to determine the role of the modified tyrosine residue at the binding site; it may also find use in a variety of applications in which controlled modulation of binding under mild conditions is required —for example, affinity chromatography ***(4)***, cell sorting or immunosensors.

1. Nitrate the antibody as described in **Subheading 3.1., steps 1–5**).
2. Perform a series of dilutions of the nitrated antibody (1:50–1:50,000; *see* **Note 5**) in 50 m*M* 2-morpholinoethane sulfonic acid (MES) saline buffer pH 5.8, and in TBS, pH 9.0.
3. Determine the binding activity of the diluted antibody at pH 5.8 and 9.0 by ELISA on microtiter plates coated with DNP-bovine serum albumin (BSA) (*see* **Note 6**).

4. Notes

1. In the first modification experiment of a protein a wide range of TNM concentrations of should be applied, for example, 0–10,000 molar excess. If loss of activity is not observed it is recommended to try again at higher pH (e.g., at pH 9.0) and with longer incubations at room temperature.
2. Although proteins hardly absorb at this 428 nm, a sample of the same concentration of the unmodified protein should be used as blank. Relatively high quantities of a protein are required for the determination of low modification ratios; for example, a single 3-nitrotyrosine per site (i.e., two per antibody molecule) would give an OD_{428nm} of approx 0.11 at antibody concentration of 2 mg/mL (13.3 m*M*).
3. Demonstrating specificity by modifying the protein in the presence of an active site specific ligand should be done under the mildest conditions that cause full loss of activity; these conditions (e.g., excess of TNM and pH) should be determined in a preliminary experiment. In some cases dialysis with 6 *M* urea (or a similar reagent) is required to release the ligand from the protein to allow the determination of its activity. In any case, control samples (without the addition of TNM) containing the protein alone and the protein incubated with the ligand should be included for comparison of the remaining activity after modification.
4. Nitrated proteins (and other chemically modified proteins as well) are often unstable. Therefore, measure the residual activity soon after modification and avoid freezing and defrosting of the samples. In those cases in which

the activity assay can be performed at low protein concentrations, for example, measuring the binding activity of an antibody by ELISA, dialysis that follows the quenching can be avoided. Protein stability can be improved by adding an equal volume of a 10 mg/mL BSA solution after the addition of the 2-mercaptoethanol.

5. To be able to observe pH-dependent binding the nitration should be performed under mild modification conditions (e.g., 200 molar excess of TNM at pH 8.3); under more extreme conditions, e.g., 1000 molar excess of TNM, the antibody is irreversibly inactivated, that is, hapten-binding is not recovered at pH 5.8 ***(2)***.
6. Conditions of the binding assay (e.g., ELISA) should be optimized to clearly identify the pH dependency of binding; in particular, the concentration of the immobilized ligand (DNP in this example) must be low enough so that the differences in binding affinities at pH 5.8 vs pH 9.0 may be observed. Thus, antigen carrying low ratios of DNP (3–10 DNPs per molecule of BSA) should be used and its concentration for the coating of the ELISA microtiter plates should be determined.

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Ethoxyformylation of Histidine

Dan S. Tawfik

1. Introduction

Diethylpyrocarbonate (DEP) reacts with various nucleophiles (amines, alcohols, thiols, imidazoles, or guanido groups) to yield the respective ethoxyformyl derivatives. At low pH (generally < 6.0) the reaction is quite selective for histidine, as the main side reaction with the ε-amino group of lysine proceeds very slowly (*see* e.g., **ref. *1***). Still, side reactions even with hydroxyl groups (e.g., of serine or tyrosine) were observed ***(2)***. The fact that the ethoxyformyl group can be removed from the imidazole side chain by mild treatment with hydroxylamine can be exploited to ascribe the modification to a histidine residue. In addition, ethoxyformylation of histidines is characterized by an increase in absorbance at 242 nm, which is also used to determine the number of modified histidines ***(1–3)***.

2. Materials

1. (DEP) (*see* **Note 1**).
2. Acetonitrile.
3. Protein for modification (approx 5 µ*M*) diluted in 0.1 *M* sodium acetate buffer, pH 5.0.
4. 1 *M* Hydroxylamine, pH 7.0.

3. Method

1. Prepare a series of *fresh* DEP solutions in acetonitrile (1–30 m*M*) (*see* **Note 2**).
2. Add 5-µL aliquots of each of the DEP solutions to 95-µL aliquots of the protein solution (*see* **Note 3**).
3. Incubate for 15–60 min.
4. Determine the activity of the modified protein.

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5. To assay the recovery of the ethoxyformylated histidine residues (*see also* **Notes 4** and **5**):
   a. Add to a solution of the modified protein 1/10 of a volume of 1 *M* hydroxylamine, pH 7.0, and incubate for 10 min.
   b. Dilute with acetate buffer and dialyze extensively against a buffer suitable for the protein being studied.
   c. Determine the activity of the protein.

4. Notes

1. The concentration of commercial DEP is often lower than indicated owing to hydrolysis. The concentration of the sample after dilution with an organic solvent can be readily determined by adding an aliquot of an imidazole solution (1–10 m*M* in phosphate, pH 7.0) and measuring the increase in absorbance at 230 nm after 5 min ($\varepsilon = 3000 M^{-1}$ cm^{-1}).
2. The final acetonitrile concentration in the protein reaction mixture should be 5< %, *see* Chapter 87, **Subheading 3.1** for typical dilutions and reaction volumes.
3. A molar excess of 10–300 of DEP is usually sufficient; however, with some proteins higher concentrations of the reagent might be needed. Likewise, if no modification is observed at pH 5.0, the reaction can be performed at higher pH. In such cases it is recommended that one ensure that loss of activity is indeed due to the ethxyformylation of histidine (*see* **Note 4**).
4. Measuring the differential UV spectra during modification with DEP is useful not only to determine the number of modified histidines but also to eliminate the possibility that residues other than histidines were modified. The ethoxyformylation of histidine side chains by DEP should result in an increase in the absorbance of the protein at 242 nm ($\varepsilon = 3200 M^{-1}$ cm^{-1}). Likewise, restoration of the activity of the modified protein after treatment with hydroxylamine should be accompanied by a parallel decrease in the absorbance at 242 nm. Ethoxyformylation of the hydroxyl of tyrosine would increase the absorbance at approx 280 nm whereas similar modification of serine or threonine does not cause a significant changes of absorbance at this range.
5. The ethoxyformyl product is quite unstable. Because the reagent is rapidly hydrolyzed (to give ethanol and carbonate) there is hardly a need to purify the protein after modification. In any case, dialysis even in neutral buffers may result in a significant removal of the ethoxyformyl group. Hence, purification by exclusion chromatography (e.g., on Sephadex G-25) is preferred.

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Modification of Arginine Side Chains with *p*-Hydroxyphenylglyoxal

Dan S. Tawfik

1. Introduction

A variety of dicarbonyl compounds including phenylglyoxal, 2, 3-butanendione, and 1, 2-cyclohexanedione selectively modify the guanidine group of arginine ***(1–3)***. The main advantage of *p*-hydroxyphenylglyoxal is in the ability to determine the number of modified arginines spectrophotometrically. This reagent is also reactive at mildly alkaline pH (usually 8.0–9.0) and yields a single product that is relatively stable ***(1)***.

2. Materials

1. *p*-Hydroxyphenylglyoxal.
2. 1 *M* NaOH.
3. Protein for modification (approx 10 µ*M*) diluted in 0.1 *M* sodium pyrophosphate buffer, pH 9.0.
4. Sephadex G-25 column.

3. Method

1. Prepare a 100 m*M* solution of *p*-hydroxyphenylglyoxal in water and adjust the pH of the solution with 1 *M* NaOH to 9.0.
2. Prepare a series of dilutions (5–50 m*M*; *see* **Note 2**) of the solution in **Step 1** in 0.1 *M* sodium pyrophosphate buffer, pH 9.0 (*see* **Note 1**).
3. Add 10-µL aliquots of the p-hydroxyphenylglyoxal solutions to 90-µL aliquots of the protein solution. Check the pH and if necessary adjust it back to pH 9.0.
4. Incubate for 60–180 min *in the dark*.
5. Pass the sample through a Sephadex G–25 column. Elute with deionized water or with an appropriate buffer (*see* **Note 4**).

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6. Determine the activity of the modified protein.
7. Determine the number of modified arginines by measuring the absorbance of the *purified* protein (*see* **Notes 3** and **4**) at 340 nm (at pH 9.0, $\varepsilon = 18{,}300\,M^{-1}\,\text{cm}^{-1}$).

4. Notes

1. An optimal rate and selectivity of modification is generally obtained at pH 8.0–9.0; however, in some proteins a higher pH might be required to modify a particular arginine residue.
2. A molar excess of *p*-hydroxyphenylglyoxal in the range of 50–500 is usually sufficient for a first trial.
3. The absorbance of *p*-hydroxyphenylglyoxal modified arginines changes with the pH. Maximal absorbance is observed at 340 nm at pH ≥ 9.0 ($\varepsilon = 18{,}300\,M^{-1}\,\text{cm}^{-1}$) ***(1)***.
4. Prolonged dialysis in neutral or mildly alkaline buffers may cause a significant release of the modifying group. Purification of the protein to determine the extent of modification should therefore be performed using exclusion chromatography ***(1)***.

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Amidation of Carboxyl Groups

Dan S. Tawfik

1. Introduction

Several reactions have been described for the modification of the carboxylic side chains of aspartic and glutamic acids ***(1,2)***; of these, amidation, using an amine and a water-soluble coupling carbodiimide reagent, is most often applied to proteins. The advantages of this approach are the stability of the modification (an amide bond) and the ability to achieve some site specificity (namely, to selectively modify a particular carboxylic side chain) by using different carbodiimide reagents and by variations in the structure (e.g., size, charge, hydrophobicity) of the amine ***(3–4)***.

The methyl or ethyl esters of glycine are commonly used as the amine nucleophile. Hydrolysis of these groups by a short treatment with 0.1 *M* hydroxyl amine (pH 8.0, 5–30 min) or a base (0.1 *M* carbonate pH 10.8, 2–6 h) affords a free carboxyl group (e.g., protein—COOH is converted into protein—CO-$NHCH_2COOH$). Hence, a mild modification that affects only the size but not the charge of the aspartyl or glutaryl side chains of the protein is achieved. Determination of the number of modified carboxylate group can be performed only by labeling the amine group, for example, by using a radiolabeled glycine ethyl ester (which is commercially available). In principle, different amines can be used to achieve selectivity or to assist the identification of the modified residues. It is important, however, to ensure that the reaction with these amines is rapid enough and yields a single product ***(3)***.

Several side reactions (e.g., with tyrosines and cysteines) may occur mainly at neutral or mildly basic pH; most of these can be ruled out by demonstrating that the activity of the modified protein is not regained after treatment with hydroxylamine.

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2. Materials

1. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (*see* **Note 1**).
2. Glycine ethyl ester.
3. Protein for modification (approx 5 µg/mL) diluted in 0.1 *M* 2-morpholinoethanesulfonic acid (MES) buffer, pH 5.5.
4. 0.1 *M* Acetate buffer, pH 5.0.

3. Method

1. Add the glycine ethyl ester to the protein solution to give a final concentration of up to 50 m*M* (*see* **Note 2**); check the pH and if necessary adjust it back to pH 5.5 (*see* **Note 3**).
2. Add EDC to a final concentration of 0.5–10 m*M* and incubate for 1–6 h (*see* **Note 4**).
3. Add one volume of acetate buffer to quench the reaction.
4. Dialyze against an appropriate buffer or pass the sample through a Sephadex G-25 column.
5. Determine the activity of the modified protein.

4. Notes

1. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is most commonly used. Several other water-soluble carbodiimide reagents are available (e.g., 1-cyclohexyl-3-[2-morpholinoethyl]carbodiimide) and can be used for this reaction; these reagents, however, are usually derived from more bulky side chains than EDC and may therefore have more limited accessibility to certain carboxylic residues of the protein.
2. Crosslinking of the protein in the presence of the coupling reagent is avoided by using relatively dilute protein solutions (≤ 0.5 mg/mL) and a large excess of the amine nucleophile (e.g., glycine ethyl ester).
3. The reaction is usually performed at acidic pH (4.5–5.5) to minimize side reactions. However, the modification of particular carboxylate side chains may require higher pH (*see* Chapter 86).
4. The reaction is usually run at ambient temperature; nevertheless, lowering the temperature to 4°C may eliminate the appearance of certain side products.

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Amidination of Lysine Side Chains

Dan S. Tawfik

1. Introduction

Perhaps the largest variety of modifications available is that for ε-amino group of lysine ***(1–4)***. The amino side chain can be acylated (using e.g., acetic anhydride) or alkylated by trinitrobenzenesulfonic acid (TNBS); these reactions alter both the size and the charge of the amino group. Other modifications, using anhydrides of dicarboxylic acids (e.g., succinic anhydride), replace the positively charged amino group with a negatively charged carboxyl group. Amidinations ***(5, 6)*** and reductive alkylations (*see* **ref. *7, 8***) offer an opportunity to modify the structure of the ε-amino group of lysines, while maintaining the positive charge. Modifications that usually do not disrupt the overall structure of the protein are preferred, particularly in those cases when one wishes to identify the specific role of lysine in the active site of the protein being studied.

Amidination is performed by reacting the protein with imidoesters such as methyl or ethyl acetimidate at basic pH. The reaction proceeds solely with amino groups to give mainly the positively charged acetimidine derivative, which is stable under acidic and mildly basic pH. Side products can be avoided by maintaining the pH above 9.5 throughout the reaction (*see* **Note 1** and **2**, **ref. *6***). The modification can be removed at a higher pH (≥ 11.0) and in the presence of amine nucleophiles (e.g., ammonia) (***5, 6***, and *see* **Note 2**).

A major drawback of this modification is that the number of amidinated lysines cannot be readily determined. However, it is possible to take advantage of the fact that the amidine group is not reactive with amine modifying reagents such as TNBS and thereby to indirectly determine the number of the remaining unmodified lysine residues after the reaction ***(9)***.

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2. Materials

1. Methyl acetimidate hydrochloride.
2. $0.1\,M$ and $1\,M$ NaOH.
3. Protein for modification.
4. $0.1\,M$ borate buffer, pH 9.5.

3. Method

1. Dissolve the protein (1–2 mg/mL) in $0.1\,M$ borate buffer pH 9.5; check the pH and if necessary adjust it back to 9.5 using $0.1\,M$ NaOH.
2. Dissolve 110 mg of methyl acetimidate hydrochloride in approx 1.1 mL of $1\,M$ NaOH (approximately $0.9\,M$); check the pH and if necessary adjust it to approx 10 with $1\,M$ NaOH (*see* **Note 1**).
2. Add an aliquot of the methyl acetimidate solution to the protein solution and check the pH again (*see* **Note 1**).
3. Incubate for 40 min.
4. Dialyze the sample against an appropriate buffer ($\text{pH} < 8.5$) or filter on a Sephadex G-25 column.
5. Determine the activity of the protein.

4. Notes

1. The amidination reaction proceeds with almost no side products only at $\text{pH} > 9.5$; at lower pH, the side reactions proceed very rapidly. Hence it is important to add the methyl acetimidine solution to the buffered protein solution without causing a change of pH ***(6)***. The methyl acetimidine is purchased as the hydrochloride salt, which is neutralized by dissolving it in $1\,M$ NaOH (*see* **Subheading 3.,** step 2). The pH of the resulting solution should be approx 10; if necessary the pH may be adjusted before the addition to the protein solution with $1\,M$ NaOH or $1\,M$ HCl. As acetimidates are rapidly hydrolyzed at basic pH, the entire process should be performed very rapidly. It is therefore recommended, in a preliminary experiment, to dissolve the methyl acetimidine hydrochloride and determine the exact amount of $1\,M$ NaOH that yields a solution of pH 10. The same process is then repeated with a freshly prepared methyl acetimidine solution which is rapidly added to the protein.
2. The acetimidyl group may be removed by treatment of the modified protein with an ammonium acetate buffer prepared by adding concentrated ammonium hydroxide solution to acetic acid to a pH of 11.3 (**Caution! Preparation of this buffer must be done carefully and in a well-ventilated chemical hood**).
3. Amidination is obviously unsuitable for the modification of proteins that are sensitive to basic pH. Reductive alkylation, using formaldehyde and sodium cyanoborohydride (*see* **ref.** ***7***), can be performed at neutral pH and is recommended for the modification of such proteins.

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Modification of Tryptophan with 2-Hydroxy-5-Nitrobenzylbromide

Dan S. Tawfik

1. Introduction

2-Hydroxy-5-nitrobenzylbromide, Koshland's reagent ***(1)***, reacts rapidly and under mild conditions with tryptophan residues. At low pH (< 7.5) this reagent exhibits a marked selectivity for tryptophan; under more basic pH or at higher reagent concentrations, cysteine, tyrosine, and even lysine residues can be modified as well (*see* **Note 1**). The reaction is extremely rapid either with the protein or with water. The reagent is relatively insoluble in water; it is therefore necessary to first dissolve it in an organic solvent (e.g., dioxane) and then to add it to the protein solution in buffer. Unlike that of most other modifying reagents, the final organic solvent concentration in the reaction mixture is relatively high (5–15%). Determination of the number of modified tryptophans is done spectrophotometrically.

2. Materials

1. 2-Hydroxy-5-nitrobenzylbromide.
2. Dioxane (water free) (*see* **Note 2**).
3. Protein for modification: 1 mg/mL in 0.1 *M* phosphate buffer, pH 7.0.
4. 1 *M* NaOH.
5. A Sephadex G-25 column.

3. Method

1. Prepare a *fresh* solution of 200 m*M* 2-hydroxy-5-nitrobenzylbromide in dioxane (keep the solution *in the dark*).
2. Dilute this solution in dioxane to 10× the final reagent concentration (*see* **Note 3**).
3. Add 10 µL of the 2-hydroxy-5-nitrobenzylbromide solution to a 90-µL aliquot of the protein solution and shake for 2 min (e.g., on a Vortex).

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4. Centrifuge (for few minutes at 10,000 rpm) if a precipitate forms (*see* **Note 4**).
5. Filter on a Sephadex G-25 and then dialyze against an appropriate buffer (*see* **Note 4**).
6. Determine the number of modified tryptophans by measuring the absorbance of the purified protein at 410 nm at pH ≥ 10 ($\varepsilon = 18{,}450\,M^{-1}\,\mathrm{cm}^{-1}$).
7. Determine the activity of the protein.

4. Notes

1. At neutral and slightly acidic pH the reaction is generally selective. At higher pH, the major side reaction is the benzylation of sulfhydryl groups; to prevent the modification of free cysteine residues the protein can be carboxymethylated prior to the modification with 2-hydroxy-5-nitrobenzylbromide.
2. Avoid the use of acetone, methanol, or ethanol as organic co-solvents for this reagent. Water-free dioxane is commercially available or can be prepared by drying dioxane over sodium hydroxide pellets.
3. At high reagent concentrations a precipitate of 2-hydroxy-5-nitrobenzyl alcohol is sometimes observed and can be removed by centrifugation. Purification of the modified protein from *all* the remaining alcohol product is sometimes difficult and may require extensive dialysis in addition to gel filtration.
4. The reaction of hydroxy-5-nitrobenzylbromide is accompanied by the release of hydrobromic acid. The pH of the reaction should be maintained if necessary by the subsequent addition of small aliquots of 1 *M* NaOH solution.

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Modification of Sulfhydryl Groups with DTNB

Dan S. Tawfik

1. Introduction

5-5 -Dithio-*bis* (2-nitrobenzoic acid) (DTNB or Ellman's reagent; **ref. *1***) reacts with the free sulfhydryl side chain of cysteine to form an S–S bond between the protein and a thionitrobenzoic acid (TNB) residue. The modification is generally rapid and selective. The main advantage of DTNB over alternative reagents (e.g., *N*-ethylmaleimide or iodoacetamide) is in the selectivity of this reagent and in the ability to follow the course of the reaction spectrophotometrically. The reaction is usually performed at pH 7.0–8.0 and the modification is stable under oxidative conditions. The TNB group can be released from modified protein by treatment with reagents that are routinely used to reduce S–S bonds, for example, mercaptoethanol, or by potassium cyanide ***(2)*** (*see* **Note 1**). In addition, the often highly pronounced differences in reactivity of different cysteine side chains in the same protein or even active site, and the availability of a variety of thiol-modifying reagents can be exploited to selectively modify cysteine side chains in proteins in the presence of other, more reactive cysteine residues ***(3)***.

2. Materials

1. DTNB.
2. 0.1 *M* Tris-HCl, pH 8.0.
3. Protein for modification: approx 5 μ*M* in 0.1 *M* Tris-HCl, pH 8.0.

3. Method

1. Prepare fresh solutions of DTNB (0.5–5 mM; see Note 2) in 0.1 M Tris-HCl, pH 8.0.
2. Add 20-μL aliquots of the DTNB solution to 180 μL of the protein solution and incubate for 30 min.

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3. Determine the number of modified cysteines by measuring the absorbance of the released TNB anion at 412 nm (= 14,150 M−1 cm−1) (*see* **Note 3**).
4. Dialyze against an appropriate buffer (*see* **Note 4**).
5. Determine the activity of the protein.

4. Notes

1. Release of the TNB modification can be achieved by treatment of the modified protein (preferably after dialysis) with thiols (e.g., mercaptoethanol) or by potassium cyanide (20 m*M* final concentration; 10–60 min). The release of the TNB anion can be followed spectrophotometrically at 412 nm ***(2)***.
2. A molar excess of 10–100 of DTNB and an incubation time of 30 min is usually sufficient with most proteins. The modification of a particular cysteine residue may require a higher DTNB concentration, a longer reaction time, or a higher pH.
3. The extent of modification is determined by measuring the absorbance of the *released* TNB anion and hence can be followed during the reaction and in the presence of an excess of unreacted DTNB.
4. Dialysis of the reaction mixture is not always necessary; in many cases the activity of the protein (enzymatic or binding) can be determined directly after the reaction (for an example *see* **ref. *2***).

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Chemical Cleavage of Proteins at Methionyl-X Peptide Bonds

Bryan John Smith

1. Introduction

One of the most commonly used methods for proteolysis uses cyanogen bromide to cleave the bond to the carboxy-(C)-terminal side of methionyl residues. The reaction is highly specific, with few side reactions and a typical yield of 90–100%. It is also relatively simple and adaptable to large or small scale. Because methionine is one of the least abundant amino acids, cleavage at that residue tends to generate a relatively small number of peptides of large size—up to 10,000–20,000 Da. For this reason the technique is usually less useful than some other methods (such as cleavage by trypsin) for identification of proteins by mass mapping, which is better done with a larger number of peptides. Cleavage at Met-X can be useful for other purposes, however:

1. Generation of internal sequence data, from the large peptides produced ***(1)***.
2. Peptide mapping.
3. Mapping of the binding sites of antibodies ***(2)*** or ligands ***(3)***.
4. Generation of large, functionally distinct domains (e.g., from hirudin, by Wallace et al. ***[4]***) or proteins of interest from fusion proteins ***(5)***.
5. Confirmation of estimates of methionine content by amino acid analysis, which has a tendency to be somewhat inaccurate for this residue ***(6)***. This is by determination of the number of peptides produced by cleavage at an assumed 100% efficiency.

2. Materials

1. Ammonium bicarbonate (0.4 *M*) solution in distilled water (high-performance liquid chromatography [HPLC] grade). Stable for weeks in refrigerated stoppered bottle.
2. 2-Mercaptoethanol. Stable for months in dark, stoppered, refrigerated bottle.

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3. Trifluoroacetic acid (TFA), Aristar grade. Make to 70% v/v by addition of distilled water (HPLC grade), and use fresh (*see* **Notes 1** and **2**).
4. CNBr. Stable for months in dry, dark, refrigerated storage. Warm to room temperature before opening. Use only white crystals, not yellow ones. **Beware of the toxic nature of this reagent: hydrogen cyanide is a breakdown product. Use in a fume cupboard (*see* Note 3)**.
5. Sodium hypochlorite solution (domestic bleach).
6. Equipment includes a nitrogen supply, fume hood, and suitably sized and capped tubes (e.g., Eppendorf microcentrifuge tubes).

3. Methods

3.1. Reduction

1. Dissolve the polypeptide in water to between 1 and 5 mg/mL, in a suitable tube. Add one volume of ammonium bicarbonate solution, and add 2-mercaptoethanol to between 1% and 5% (v/v).
2. Blow nitrogen over the solution to displace oxygen, seal the tube, and incubate at room temperature for approx 18 h.

3.2. Cleavage

1. Dry down the sample under vacuum, warming if necessary to help drive off all of the bicarbonate. Any remaining ammonium bicarbonate will form a nonvolatile salt on subsequent reaction with the TFA that follows. If a white salty deposit remains, redissolve in water and dry down again.
2. Redissolve the dried sample in 70% v/v TFA, to a concentration of 1–5 mg/mL.
3. Add excess white crystalline CNBr to the sample solution, to 10-fold or more molar excess over methionyl residues. Practically, this amounts to approx equal weights of protein and CNBr. To very small amounts of protein, add one small crystal of reagent. Carry out this stage in the fume hood (*see* **Notes 3** and **4**).
4. Seal the tube and incubate at room temperature for 24 h.
5. Terminate the reaction by drying down under vacuum. Store samples at −10°C or use immediately (*see* **Note 5**).
6. Immediately after use decontaminate equipment that has contacted CNBr by immersion in hypochlorite solution (bleach) until effervescence stops (a few minutes). Wash decontaminated equipment thoroughly.

4. Notes

1. The mechanism of the action of cyanogen on methionine-containing peptides is shown in **Fig. 1**. For further details, see the review by Fontana and Gross *(7)*. The methioninyl residue is converted to homoseryl or homoseryl lactone. The relative amounts of these two depend on the acid used, but when 70% TFA is the solvent, homoserine lactone is the major derivative. Peptides generated are suitable for peptide sequencing by Edman

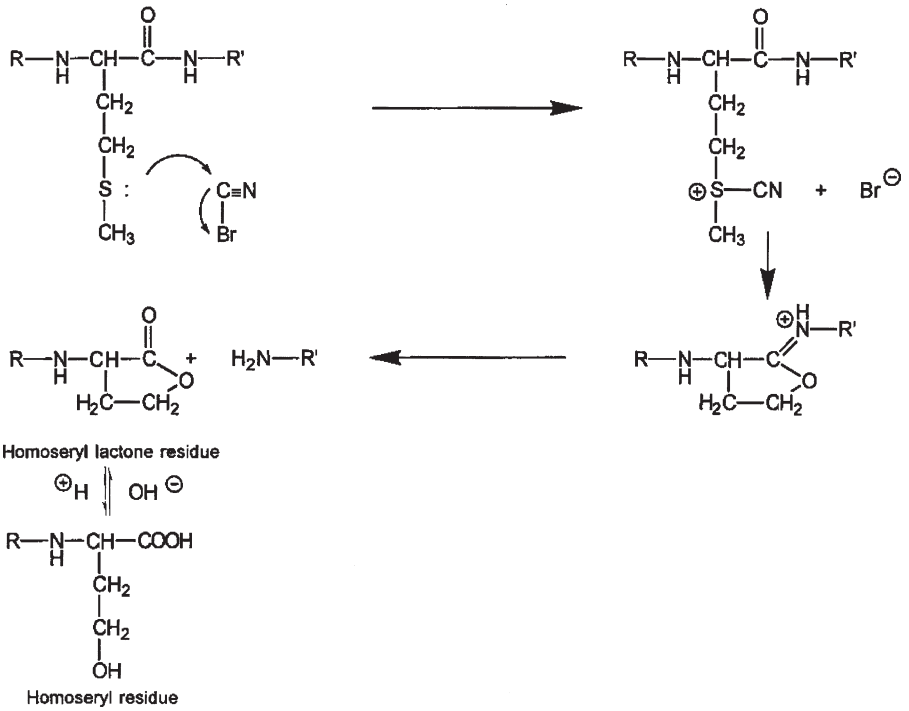

Fig. 1. Mechanism of cleavage of Met-X bonds by CNBr.

chemistry. Methionine sulfoxide does not take part in this reaction and the first step in the method is intended to convert any methionyl sulfoxide to methionyl residues, and so maximize cleavage efficiency. If the reduction is not carried out, the efficiency of cleavage may not be greatly diminished. If virtually complete cleavage is not necessary, partial cleavage products are desired (*see* **Note 6**), the sample is small and difficult to handle without loss, or speed is critical, the reduction step may be omitted. An acid environment is required to protonate basic groups and so prevent reaction there and maintain a high degree of specificity. Met-Ser and Met-Thr bonds may give significantly less than 100% yields of cleavage and simultaneous conversion to methionyl to homoseryl residues within the uncleaved polypeptide. This is because of the involvement of the β-hydroxyl groups of seryl and threonyl residues in alternative reactions, which do not result in cleavage *(7)*. Morrison et al. *(8)* however, have found that use of 70% v/v TFA gives a better yield of cleavage of a Met-Ser bond in apolipoprotein A1 than does use of 70% formic acid (*see* **Note 2**). Using model peptides, Kaiser and Metzka *(9)* analyzed the cleavage reaction at Met-Ser and Met-

Thr and concluded that cleavage that efficiency is improved by increasing the amount of water present, and for practical purposes 0.1 *M* HCl is a good acid to use, giving about 50% cleavage of these difficult bonds. Remaining uncleaved molecules contained either homoserine or methionyl sulfoxide instead of the original methionyl. Cleavage efficiency improved with increasing strength of acid, but there was an accompanying risk of degradation in the stronger acids. Utilization of C-terminal homoseryl lactone for linkage to solid phase is discussed in **Note 7**.

2. Acid conditions are required for the reaction to occur. In the past, 70% v/v formic acid (pH 1) was commonly used because it is a good protein solvent and denaturant, and also volatile. However, it may damage tryptophan and tyrosine residues ***(8)*** and also cause formation of seryl and threonyl side chains (showing up during analysis by mass spectroscopy as an increase of 28 amu per modification ***[9,10]***). Use of other acids avoids this problem. TFA (also volatile) may be used in concentrations in the range 50%–100% (v/v). The pH of such solutions is approx pH 0.5 or less. The rate of cleavage in 50% TFA may be somewhat slower than in 70% formic acid, but similar reaction times of hours, up to 24 h will provide satisfactory results. Caprioli et al. ***(11)*** and Andrews et al. ***(12)*** have illustrated the use of 60% and 70% TFA (respectively) for cyanogen bromide cleavage of proteins. Acetic acid (50%–100% v/v) may be used as an alternative but reaction is somewhat slower than in TFA. Alternatively, 0.1 *M* HCl has been used ***(9,10)***. To increase solubilization of proteins, urea or guanidine·HCl may be added to the solution. Thus, in 0.1 *M* HCl, 7 *M* urea, for 12 h at ambient temperature, a Met-Ala bond was cleaved with 83% efficiency, and the more problematical Met-Ser and Met-Thr bonds with 56% and 38% efficiency, respectively ***(9)***.
3 Although the specificity of this reaction is excellent, some side reactions may occur. This is particularly so if colored (yellow or orange) CNBr crystals are used, when there may be destruction of tyrosyl and tryptophanyl residues and bromination may also be detected by mass spectroscopy. Treatment of samples in other formats is discussed in **Notes 8** and **10**.
4. The above protocol describes addition of solid CNBr to the acidic protein solution, to give a molar excess of CNBr over methionyl residues. This has the advantage that pure white crystals may be selected in favor of pale yellow ones showing signs of degradation (*see* **Note 3**). It does not allow accurate estimation of the quantity of reagent used, however. The work of Kaiser and Metzka ***(9)*** suggests that more than a 10-fold molar excess of CNBr over methionyl residues does not increase the extent of cleavage. If in doubt as to the concentration of methionyl residues, however, err on the side of higher cyanogen concentration. If accurate quantification of CNBr is required, solid cyanogen bromide may be weighed out and dissolved to a given concentration by addition of the appropriate volume of 70% v/v

TFA, and the appropriate volume of that solution added to the sample. The CNBr will start to degrade once in aqueous acid, so use when fresh. An alternative is to dissolve the CNBr in acetonitrile, in which it is more stable. CNBr in acetonitrile solution is available commercially, for instance, at a concentration of 5 *M* (Aldrich). While such a solution may be seen to be degrading by its darkening color, this is not so obvious as it is with CNBr in solid form. For use, sufficient acetonitrile solution is added to the acidic protein solution to give the desired excess of cyanogen bromide over protein (e.g., 1/20 dilution of a 5 *M* CNBr solution to give a final 250 m*M* solution). The data of Kaiser and Metzka ***(9)*** indicate that high concentrations (70–100%) of acetonitrile can interfere with the cleavage reaction by decreasing the amount of water present, but below a concentration of 30% (in 0.1 *M* HCl) the effect is noticeable in causing a small decrease of Met-Ser and Met-Thr bond cleavage, but negligible for the Met-Ala bond.

5. The reagents used are removed by lyophilization, unless salt has formed following failure to remove all the ammonium bicarbonate. The products of cleavage may be fractionated by the various forms of electrophoresis and chromatography currently available. If analyzed by reverse phase HPLC, the reaction mixture may be applied to the column directly without lyophilization. Since methionyl residues are among the less common residues, peptides resulting from cleavage at Met-X may be large and therefore in HPLC, use of wide-pore column materials may be advisable (e.g., 30 µm pore size reverse-phase column, using gradients of acetonitrile in 0.1% v/v TFA in water). Beware that some large peptides that are generated by this technique may prove to be insoluble (fore instance, if the solution is neutralized after the cleavage reaction) and therefore form aggregates and precipitates.
6. Incomplete cleavage, generating combinations of (otherwise) potentially cleaved peptides, may be advantageous, for ordering peptides within a protein sequence. Mass spectrometric methods are suitable for this type of analysis ***(10)***. Such partial cleavage may be achieved by reducing the duration of reaction, even to less than 1 h ***(10)***. The acid conditions employed for the reaction may lead to small degrees of deamidation of glutamine and asparagine side chains (which occurs below pH 3) and cleavage of acid-labile bonds, for example, Asp-Pro. A small amount of oxidation of cysteine to cysteic acid may occur, if these residues have not previously been reduced and carboxymethylated. Occasional cleavage of Trp-X bonds may be seen, but this does not occur with good efficiency, as it does when the reduction step of this technique is replaced by an oxidation step (for a description of this approach to cleavage of Trp-X bonds). Rosa et al. ***(13)*** cleaved both Met-X and Trp-X bonds simultaneously by treatment of protein with 12 m*M* CNBr in 70% TFA solution, plus 240 µM potassium bromide.

7. As described in **Note 1**, the peptide to the N-terminal side of the point of cleavage, has at its C-terminus a homoserine or homoserine lactone residue. The lactone derivative of methionine can be coupled selectively and in good yield ***(17)*** to solid supports of the amino type, for example, 3-amino propyl glass. This is a useful technique for sequencing peptides on solid supports. The peptide from the C-terminus of the cleaved protein will, of course, not end in homoserine lactone (unless the C-terminal residue was methionine!) and so cannot be so readily coupled. Similarly, the C-terminal peptide carboxyl can react (if not amidated) with acidic methanol, to become a methyl ester (with a corresponding mass increment of 14 amu). Homoserine lactone, present as the C-terminal residue on other peptides in a CNBr digest, will react with acidic methanol and show a mass increase of 32 amu. With account made for side chain carboxyl residues, this is a means to identify C-terminal peptides by mass spectroscopy ***(18)***.
8. Frequently, the protein of interest is impure, in a preparation containing other proteins. Polyacrylamide gel electrophoresis (PAGE) is a popular means by which to resolve such mixtures. Proteins in gel slices may be subjected to treatment with CNBr ***(14)***, as follows: The piece of gel containing the band of interest is cut out, lyophilized, and then exposed to vapor from a solution of CNBr in TFA for 24 h, at room temperature in the dark. The vapor is generated from a solution of 20 mg CNBr in 1 mL of 50% v/v TFA by causing it to boil by placing it under reduced pressure, in a sealed container together with the sample. The gel piece is then lyophilized again, and the peptides in it analyzed by PAGE.
9. Proteins that have been transferred from polyacrylamide gel to polyvinylidene difluoride (PVDF) membrane may be cleaved *in situ*, as described by Stone et al. ***(15)***. The protein band (of a few micrograms) is first cut from the membrane on the minimum size of PVDF (as excess membrane reduces final yield). The dried membrane is then wetted with about 50 µL of CNBr solution in acid solution—Stone et al. ***(15)*** report the use of CNBr applied in the ratio of about 70 µg per 1 g of protein. Note that although PVDF does not wet directly in water, it *does* do so in 70% formic acid, or in the alternative of 50% or 70% v/v TFA. Cleavage is achieved by incubation at room temperature, in the dark for 24 h. Oxidation of methionine during electrophoresis and blotting was not found to be a significant problem in causing reduction in cleavage yield, being about 100% in the case of myoglobin ***(15)***. The peptides generated by cleavage may be extracted for further analysis, first in the solution of CNBr in the acid solution, second in 100 µL of acetonitrile (40% v/v, 37°C, 3 h), and thirdly in 100 µL of TFA (0.05% v/v in 40% acetonitrile, 50°C. All extracts are pooled and dried under vacuum before any subsequent analysis.

10. Analysis of protein samples in automated peptide sequencing may sometimes yield no result. The alternative causes (lack of sample or amino-(N)-terminal blockage) may be tested by cleavage at methionyl residues by CNBr. Generation of new sequence(s) indicates blockage of the original N-terminus. The method is similar if the sample has been applied to a glass fiber disk, or to a piece of PVDF membrane in the sequencing cartridge. The cartridge containing the filter and/or membrane is removed from the sequencer, and the filter and/or membrane saturated with a fresh solution of CNBr in acid solution. The cartridge is wrapped in sealing film to prevent drying out, and then incubated in the dark at room temperature for 24 h. The sample is then dried under vacuum, replaced in the sequencer and sequence started again. Yields tend to be poorer than in the standard method described in the preceding. If the sample contains more than one methionine, more than one new N-terminus is generated, leading to a complex of sequences. This may be simplified by subsequent reaction with orthophthaladehyde which blocks all N-termini except those bearing a prolyl residue ***(16)***. For success with this approach, prior knowledge of the location of proline in the sequence is required, the reaction with orthophthaladehyde being conducted at the appropriate cycle of sequencing.
11. The reagents used are removed by lyophilization, unless salt has formed following failure to remove all of the ammonium bicarbonate. The products of cleavage may be fractionated by the various forms of electrophoresis and chromatography currently available. If analyzed by reverse-phase HPLC, the reaction mixture may be applied to the column directly without lyophilization. As methionyl residues are among the less common residues, peptides resulting from cleavage at Met-X may be large, and so in HPLC use of wide-pore column materials may be advisable (e.g., 30-µm pore size reverse-phase columns, using gradients of acetonitrile in 0.1% v/v TFA in water). Beware that some large peptides that are generated by this technique may prove to be insoluble (e.g., if the solution is neutralized after the cleavage reaction) and so form aggregates and precipitates.

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Chemical Cleavage of Proteins at Tryptophanyl-X Peptide Bonds

Bryan John Smith

1. Introduction

Tryptophan is represented in the genetic code by a single codon and has proven useful in cloning exercises in providing an unambiguous oligonucleotide sequence as part of a probe or primer. It is also one of the less abundant amino acids found in polypeptides, and cleavage of bonds involving tryptophan generates large peptides. This may be convenient for generation of internal sequence information (the usual purpose to which this technique is put), but is less useful for identification of proteins by mass mapping, where a larger number of smaller peptides makes for more successful database searching. Development of mass spectrometric approaches (determination of short sequences from short peptides) in recent years has meant that methods of cleavage at Tryptophanyl residues have become used more rarely.

While there is no protease that shows specificity for tryptophanyl residues, various chemical methods have been devised for cleavage of the bond to the carboxy-(C)-terminal side of tryptophan. These are summarized in **Table 1**. Some show relatively poor yields of cleavage, and/or result in modification of other residues (such as irreversible oxidation of methionine to its sulfone), or cleavage of other bonds (such as those to the C-terminal side of tyrosine or histidine). The method described in this chapter is one of the better ones, involving the use of cyanogen bromide (CNBr). Cleavage of bonds to the C-terminal side of methionyl residues (*see* Chapter 94) is prevented by prior reversible oxidation to methionine sulphoxide. Cleavage by use of *N*-bromosuccinimide or *N*-chlorosuccinimide remains a popular method, however, despite some chance of alternative reactions (*see* **Table 1**).

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Table 1
Summary of Methods for Cleavage of Trp-X Bonds in Polypeptides

Brief method details[a]	Comments	Example ref.
1. Incubation in molar excess of cyanogen bromide: a. Incubation in glacial acetic acid: 9 *M* HCl (2/1 [v/v] with DMSO, room temperature, 30 min. b. Neutralization c. Incubation with CNBr in acid (e.g., 60% formic acid) 4°C, 30 h in the dark.	*See text* for further details. Yields and specificity excellent; Met oxidized to sulfoxide by step a.	*4*
2. Incubation in very large (up to 10,000-fold) molar excess of CNBr over Met, in heptafluorobutyric acid–88%–HCOOH (1/1 [v/v]), room temperature, 24 h in the dark.	To inhibit Met-X cleavage, Met is photooxidized irreversibly to Met sulfone; yield poor.	*8*
3. a. Incubate protein for 30 min, room temperature, in: 21.6 mg/mL of phenol in glacial acetic acid 12 *M* HCl–DMSO:24:12:1,	Cys and Met oxidized; some deamidation and cleavage around Asp may occur; fresh, colorless HBr required.	*9*
b. Add 0.1 vol of 48% HBr, 0.03 by volume of DMSO, and incubate for 30 min, room temperature.	Trp converted to dioxindolylalanyl lactone. Protein containing dioxindolylalanine derivatives(s) but remaining uncleaved may be cleaved to improve yield to approx 80% as follows: *Incubate in* 10% acetic acid, 60°C 10 –15 h.	
4. Incubate in BNPS-skatole[b] 100-fold molar excess over Trp) in 50% acetic acid (v/v) room temperature, 48 h, in the dark.	Reagent is unstable—fresh reagent required to minimize side reactions; some reactions with Tyr may occur, but addition of free Tyr to reaction mixture minimizes this; Met and Xys may be oxidized, yields up to 60%.	*10*

	Alternative conditions for rapid reaction: neat acetic acid 47°C 15–60 min; adaptable for cleavage of proteins bound to glass fiber or PVDF[c].	*11*
5. Incubation in *N*-bromosuccinimide (NBS, 3-fold molar excess over His, Trp, and Tyr), pH 3.0–4.0 (e.g., pyridine–acetic acid pH 3.3) 1 h, 100°C.	Also less rapid cleavage at His-X and Tyr; yields moderate to poor; Trp converted to lactone derivative.	*12*
6. Incubation in *N*-chlorosuccinimide (NCS,10-fold molar excess) 13 over (protein) in 27.5% acetic acid, 4.68 *M* urea; room temperature, 30 min; stopped by addition of *N*-acetyl-L-methionine.	Yield approx 50%; oxidation of methionine (to sulfone) and (to cysteic acid), especially in higher concentrations; *N*-chloro–succinate; Tyr and His not modified or cleaved (cf. *N*-bromo–succinimide; method 5); adaptable for cleavage of proteins in gels.	*13* *14*
7. Incubate in 80% acetic acid containing 4 *M* guanidine; HCl, 13 mg/mL iodosobenzoic acid; 20 mL *p*-cresol, for 20 h, room temperature, in the dark.	Specificity and yields good; the *p*-cresol is used to prevent cleavage at Tyr; Trp converted to lactone derivative.	*15*

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[a] All methods cleave bond to the C-terminal side of residue.

[b] BNPS-skatole = 3-bromo-3-methyl-2-(2'-nitrophenylsulfenyl) indolenine.

[c] PVDF = polyvinylidene difluoride.

Apart from its use in peptide mapping and generation of peptides for peptide sequencing, cleavage at tryptophan residues has also been used to generate peptides used to map the binding site of an antibody ***(1)***, or the sites of phosphorylation ***(2)***. Another application has been generation of a recombinant protein from a fusion protein ***(3)***: tryptophan was engineered at the end of a β-galactosidase leader peptide, adjacent to the N-terminus of phospholipase A_2. Reaction with *N*-chlorosuccinimide allowed subsequent purification of the enzyme without leader peptide.

2. Materials

1. Oxidizing solution: Mix together 30 vol of glacial acetic acid, 15 vol of 9 *M* HCl, and 4 vol of dimethyl sulfoxide (DMSO). Use best grade reagents. Although each of the constituents is stable separately, mix and use the oxidizing solution when fresh.
2. Ammonium hydroxide (15 *M*) (*see* **Note 4**).
3. CNBr solution in formic acid (60% v/v): Bring 6 mL of formic acid (minimum assay 98%, Aristar grade) to 10 mL with distilled water. Add white crystalline cyanogen bromide to a concentration of 0.3 g/mL. Use when fresh (*see* **Note 5**.) Store CNBr refrigerated in the dry and dark, where it is stable for months. Use only white crystals. **Beware of the toxic nature of this reagent. Use in a fume hood**.
4. Sodium hypocholorite solution (domestic bleach).
5. Equipment includes a fume hood and suitably sized capped tubes (e.g., Eppendorf microcentrifuge tubes).

3. Methods

1. Oxidation: Dissolve the sample to approx 0.5 nmol/μL in oxidizing solution (e.g., 2–3 nmol in 4.9 μL of oxidizing solution). Incubate at 4 °C for 2 h (*see* **Notes 6** and **7**).
2. Partial neutralization: To the cold sample, add 0.9 volume of ice-cold NH_4OH (e.g., 4.4 μL of NH_4OH to 4.9 μL of oxidized sample solution). Make this addition carefully so as to maintain a low temperature (*see* **Note 7**.)
3. Cleavage: Add 8 vol of CNBr solution. Incubate at 4 °C for 30 h in the dark. Carry out this step in a fume hood.
4. To terminate the reaction, lyophilize the sample (all reagents are volatile). (*See* **Note 8**).
5. Decontaminate equipment such as spatulas that have contacted CNBr, by immersion in bleach until the effervescence stops (a few minutes) and thorough washing.

4. Notes

1. The method described is that of Huang et al. ***(4)***. Although full details of the mechanism of this reaction are not clear, it is apparent that tryptophanyl residues are converted to oxindolylalanyl residues in the oxidation step, and the bond to the C-terminal side at each of these is readily

cleaved in excellent yield (approaching 100% in **ref. *4***) by the subsequent CNBr treatment. The result is seemingly unaffected by the nature of the residues surrounding the cleavage site. During the oxidation step, methionyl residues become protected by conversion to sulfoxides, bonds at these residues not being cleaved by the cyanogen bromide treatment. Cysteinyl residues will also suffer oxidation if they have not been reduced and alkylated beforehand. Rosa et al. ***(5)*** cleaved both Trp-X and Met-X bonds simultaneously by omission of the oxidation step and inclusion of 240 µ*M* potassium iodide in the reaction of protein with 12 m*M* CNBr in 70% TFA solution. The peptide to the C-terminal side of the cleavage point has a free amino-(N)-terminus and so is suitable for amino acid sequencing by Edman chemistry.

2. Methionyl sulfoxide residues in the peptides produced may be converted back to the methionyl residues by incubation in aqueous solution with thiols (e.g., dithiothreitol, as described in **ref. *3***, or see use of 2-mercaptoethanol above).
3. The acid conditions used for oxidation and cleavage reactions seem to cause little deamidation ***(4)***, but one side reaction that can occur is hydrolysis of acid-labile bonds. The use of low temperature minimizes this problem. If a greater degree of such acid hydrolysis is not unacceptable, speedier and warmer alternatives to the reaction conditions described above can be used as follows:

    a. Oxidation at room temperature for 30 min, but cool to 4 °C before neutralization.
    b. Cleavage at room temperature for 12–15 h.

4. As alternatives to the volatile base NH_4OH, other bases may be used (e.g., the nonvolatile potassium hydroxide or Tris base).
5. Formic acid is a good protein denaturant and solvent, and is volatile and so relatively easy to remove. However, it has been noted that use of formic acid can cause formulation of seryl and threonyl residues ***(6)*** in the polypeptide (seen as an 28 amu increase in molecular mass) and damage to tryptophan and tyrosine (evidenced by spectral changes ***[7]***). As an alternative to formic acid, 5 *M* acetic acid may be used, or as in the use of CNBr in cleaving methionyl-X bonds, 70% (v/v) trifluoroacetic acid may prove an acceptable alternative ***(5)***.
6. Samples eluted from sodium dodecylsulfate (SDS) gels may be treated as described, but for good yields of cleavage, Huang et al. (4) recommend that the sample solutions are acidified to pH 1.5 before lyophilization in preparation for dissolution in the oxidizing solution. Any SDS present may help to solubilize the substrate and, in small amounts at least, does not interfere

with the reaction. However, nonionic detergents that are phenolic or contain unsaturated hydrocarbon chains (e.g., Triton, Nonidet P-40) and reducing agents are to be avoided.

7. The method is suitable for large-scale protein cleavage, requiring simple scaling up. Huang et al. ***(4)*** made two points, however:
   a. The neutralization reaction generates heat. As this might lead to protein or peptide aggregation, cooling is important at this stage. Ensure that the reagents are cold and are mixed together slowly and with cooling. A transient precipitate is seen at this stage. If the precipitate is insoluble, addition of SDS may solubilize it (but will not interfere with the subsequent treatment).
   b. The neutralization reaction generates gases. Allow for this when choosing a reaction vessel.
8. At the end of the reaction, all reagents may be removed by lyophilization and the peptide mixture analyzed, for example, by polyacrylamide gel electrophoresis or by reverse-phase high-performance liquid chromatography (HPLC). Peptides generated may tend to be large, ranging up to a size of the order of 10,000 Da or more. Some of these large peptides may not be soluble, for example, if the solution is neutralized following the cleavage reaction, and consequently they aggregate and precipitate.
9. Note that all reactions are performed in one reaction vial, eliminating transfer of sample between vessels, and so minimizing peptide losses that can occur in such exercises.
10. Alternative methods for cleavage of tryptophanyl-X bonds are outlined in **Table 1**. The method (vi) that employs *N*-chlorosuccinimide is the most specific but shows only about 50% yield. BNPS-skatole is a popular Trp-X-cleaving reagent whose reaction and products have been studied in some detail (e.g., *see* **refs. *16*** and ***17***)
11. Both BNPS-skatole and N-chlorosuccinimide methods (**Table 1**, iv and vi, respectively) have been adapted to cleave small amounts (micrograms or less) of proteins on solid supports or in gels. Thus, proteins bound to glass fiber (as used in automated peptide sequences) may be cleaved by wetting the glass fiber with 1 μg/mL BNPS-skatole in 70%, (v/v) acetic acid, followed by incubation in the dark for 1 h at 47 °C. After drying, sequencing may proceed as normal. Alternatively protein blotted to polyvinylidene difluoride membrane may be similarly treated and resulting peptides eluted for further analysis ***(11)***. Alternatively, protein in slices of polyacrylamide gel (following polyacrylamide gel electrophoresis [SDS–PAGE]) may be cleaved by soaking for 30 min 0.015 *M* in N-chlorosuccinimide, in 0.5 g/mL of urea in 50%, (v/v) acetic acid. Following washing, peptides may be electrophoresed to generate peptide maps ***(14)***.

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Chemical Cleavage of Proteins at Aspartyl-X Peptide Bonds

Bryan John Smith

1. Introduction

Some methods for chemically cleaving proteins, such as those described in Chapters 94 and 95, are fairly specific for a particular residue, show good yields, and generate usefully large peptides (as reaction occurs at relatively rare amino acid residues). There may be cases, however, when such peptides (or indeed, proteins) lacking these rarer residues need to be further fragmented, and in such instances cleavage at the more common aspartyl residue may prove useful. The method described in this chapter (and in **ref.** ***1***) for cleavage to the carboxy-(C)-terminal side of aspartyl residues is best limited to smaller polypeptides rather than larger proteins because yields are <100% and somewhat variable according to sequence. Partial cleavage of Asp-X bonds in a larger protein leads to a very complex set of peptides that may be difficult to analyze. Partial hydrolysis of smaller peptides yields correspondingly simpler mixtures. This may even be preferable to complete fragmentation for some purposes, such as peptide sequencing or mass spectrometry whereby the series of overlapping peptides may be used to order the peptides in the protein sequence (as in **ref.** ***2*** in which partial cleavage at methionyl-X bonds by CNBr was used).

2. Materials

1. Dilute HCl (approx 0.013 *M*) pH 2 ± 0.04: Dilute 220 µL of constant boiling (6 *M*) HCl to 100 mL with distilled water.
2. Pyrex glass hydrolysis tubes.
3. Equipment includes a blowtorch suitable for sealing the hydrolysis tubes, a vacuum line, and an oven for incubation of samples at 108°C.

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3. Method

1. Dissolve the protein or peptide in the dilute acid to a concentration of 1–2 mg/mL in a hydrolysis tube.
2. Seal the hydrolysis tube under vacuum; that is, with the hydrolysis (sample) tube connected to a vacuum line, using a suitably hot flame, draw out and finally seal the neck of the tube.
3. Incubate at 108°C for 2 h *(see* **Note 5**).
4. To terminate the reaction, cool and open the hydrolysis tube, dilute the sample with water, and lyophilize.

4. Notes

1. The bond most readily cleaved in dilute acid is the Asp-X bond, by the mechanism outlined in **Fig. 1(A)**. The bond X-Asp may also be cleaved, in lesser yields (*see* **Fig. 1[B]**). Thus, either of the peptides resulting from any one cleavage may keep the aspartyl residue at the point of cleavage, or neither might, if free aspartic acid is generated by a double cleavage event. Any of these peptides is suitable for sequencing.
2. The amino acid sequence of the protein can affect the lability of the affected bond. Thus, the aspartyl-prolyl bond is particularly labile in acid conditions

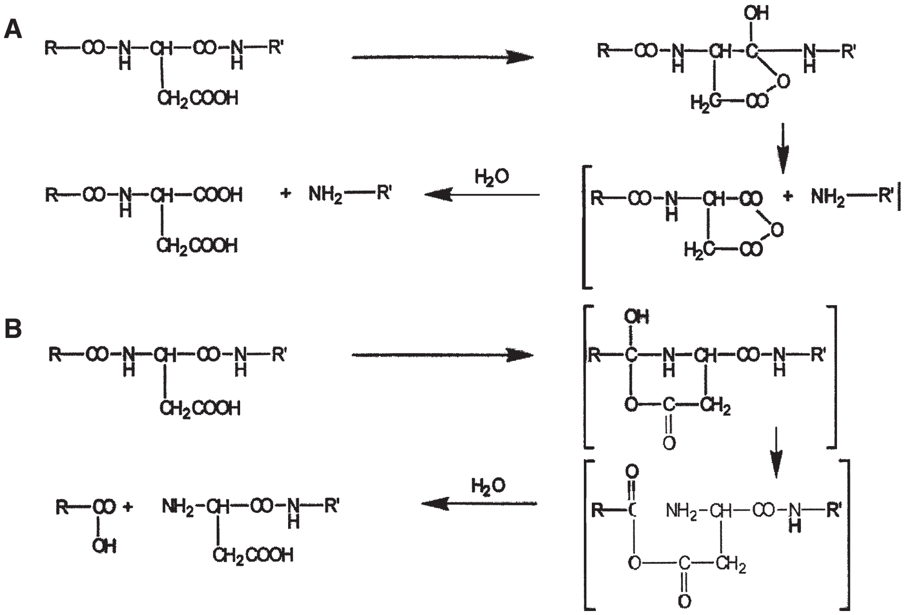

Fig. 1 Mechanisms of the cleavage of bonds to the COOH site (scheme A) and to the NH2 site (scheme)

(*see* **Note 3**). Ionic interaction between the aspartic acid side chains and basic residue side chains elsewhere in the molecule can adversely affect the rate of cleavage at the labile bond. Such problems as these make prediction of cleavage points somewhat difficult, particularly if the protein is folded up (e.g., a native protein). The method may well prove suitable for use in cleaving small proteins or peptides, in which such intramolecular interactions are less likely. Nevertheless, yields are <100%—up to about 70% have been reported ***(1)***.

3. As noted in the preceding the aspartyl-prolyl bond is particularly acid labile and the following conditions have been proposed to promote cleavage of this particular bond ***(3)***: dissolution of the sample in guanidine·HCl (7*M*) in dilute acid (e.g., acetic acid, 10% v/v, adjusted to pH 2.5 with pyridine); incubation at moderate temperature (e.g., 37°C) for prolonged periods (e.g., 24 h); terminate by lyophilization. Inclusion of guanidine. HCl, intended to denature the protein, may still fail to render all aspartyl-prolyl bonds sensitive to cleavage.
4. The conditions of low pH can be expected to cause a number of side reactions: cleavage at glutamyl residues; deamidation of (and possibly some subsequent cleavage at) glutaminyl and asparaginyl residues; partial destruction of tryptophan; cyclization of amino-(N)-terminal glutaminyl residues to residues of pyrrolidone carboxylic acid; α-β shift at aspartyl residues. The last two changes create a blockage to Edman degradation. The short reaction time of 2 h is intended to minimize these side reactions. A small degree of loss of formyl or acetyl groups from N-termini ***(1)*** is another possible side reaction but is not recognized as a significant problem, generally.
5. A polypeptide substrate that is insoluble in cold dilute HCl may dissolve during the incubation at 108°C. Formic acid is a good protein denaturant and solvent and may be used instead of HCl as follows: dissolve the sample in formic acid (minimum assay 98%, Aristar grade), then dilute 50–fold to pH 2; proceed as in method for HCl. Note, however, that incubation of protein in formic acid may result in formylation (increased molecular mass ***[2]***) and damage to tryptophan and tyrosine residues (altered spectral properties ***[4]***).
6. The comments concerning the effect of the amino acid sequence and of the environment around potentially labile bonds, and the various side reactions that can occur, indicate that the consequences of incubation of a protein in dilute acid are difficult to predict—they are best investigated empirically by monitoring production of peptides by electrophoresis or high-performance liquid chromatography (HPLC).
7. The method described has the benefit of simplicity. It is carried out in a single reaction vessel, with reagents being removed by lyophilization at the

end of reaction. Thus, sample handling and losses incurred during this are minimized. This makes it suitable for subnanomolar quantities of protein, although the method may be scaled up for larger amounts also.

8. Note that bonds involving aspartyl residues may also be cleaved by commercially available enzymes. Endoproteinase Asp-N hydrolyzes the bond to the N-terminal side of an aspartyl residue, but also of a cysteinyl residue. Glu-C cleaves the bond to the C-terminal side of glutamyl and aspartyl residues.
9. The literature has various examples of unwanted cleavage of Asp-X bonds as the result of incubation of protein in acid conditions. The conditions encountered during N-terminal sequencing by Edman chemistry cause cleavage at Asp-X, generally somewhat inefficiently but sufficient to cause gradually increasing background. Cleavage may occur during protein isolation, for instance, of guanylin by 1 *M* acetic acid, which causes artificial cleavage from its corresponding prohormone ***(5)***. Cleavage of labile bonds may also occur during heating of samples in sodium dodecyl sulfate (SDS) solution in preparation for SDS-polyacrylamide gel elctrophoresis (SDS-PAGE) ***(6)***. Cleavage of Asp-X bonds has also been noted to occur in the process of matrix-assisted mass spectrometry ***(7)***.

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Chemical Cleavage of Proteins at Cysteinyl-X Peptide Bonds

Bryan John Smith

1. Introduction

Cysteine is a significant amino acid residue in that it can form a disulfide bridge with another cysteine (to form cystine). Such disulfide bridges are important determinants of protein structure. No known endoproteinase shows specificity solely for cysteinyl, or cystinyl, residues, although endoproteinase Asp-N is able to hydrolyze bonds to the amino-(N)-terminal side of aspartyl or cysteinyl residues. Modification of aspartyl residues ***(1)*** can generate specificity for cysteinyl residues. Again, as discussed by Aitken ***(2)***, modification of cysteinyl to 2-aminoethylcysteinyl residues makes the bond to the carboxy-(C)-terminal side susceptible to cleavage by trypsin, or the bond to the N-terminal side sensitive to Lys-N. However, specific cleavage of bonds to the N-terminal side of cysteinyl residues may be achieved in good yield by chemical means [***(3)***; *see* **Note 1**]. The cleavage generates a peptide blocked at its N-terminus as the cysteinyl residue is converted to an iminothiazolidinyl residue, but peptide sequencing can be carried out after conversion of this residue to an alanyl residue ***(4)***.

Cysteine is one of the less frequent amino acids in proteins, and cleavage at cysteinyl residues tends to generate relatively large peptides. Thus, the method described in this chapter has been used to generate separate domains of troponin C for the purpose of studying Ca and peptide binding ***(5)***, and peptides from vinculin to study talin- and anti-vinculin antibody binding ***(6)***. It has also been adapted for the purposes of "footprinting": Cys residues that remain protected in a native protein remain noncyanylated on reaction with 2-nitro-5-thiocyanobenzoate, and remain uncleaved upon alteration of the pH to 9.0. Thus the protected Cys residues can be mapped within the proteins sequence ***(7)***.

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2. Materials

1. Modification buffer: 0.2 *M* Tris-acetate, pH 8.0, 6 *M* guanidine.HCl, 10 m*M* dithiothreitol. Use Analar grade reagents and high-performance liquid chromatography (HPLC)-grade water. (*See* **Note 4.**)
2. 2-Nitro-5-thiocyanobenzoate (NTCB): Commercially available (Sigma) as a yellowish powder. Contact with skin, eyes, etc. may cause short-term irritation. Long-term effects are unknown, so handle with care (wear protective clothing). Sweep up spillages. Store at 0–5 °C.
3. NaOH solution, sufficiently concentrated to allow convenient alteration of reaction pH. For example: 2 *M* in HPLC-grade water.
4. Deblocking buffer: 50 m*M* Tris-HCl, pH 7.0.
5. Raney nickel activated catalyst: Commercially available (e.g., from Sigma as 50% slurry in water, pH >9). Wash in deblocking buffer prior to use. A supply of N_2 gas is also required for use with the Raney nickel. (*See* **Note 7**.)

3. Methods (*see* Note 3)

1. Dissolve the polypeptide to a suitable concentration (say, 2 mg/mL) in the modification buffer (pH 8.0). To reduce disulfides in the dithiothreitol, incubate at 37 °C for 1–2 h. (*See* **Note 4**.)
2. Add NTCB to 10-fold excess over sulfhydryl groups in polypeptide and buffer. Incubate at 37 °C for 20 min.
3. To cleave the modified polypeptide, adjust to pH 9 by addition of NaOH solution. Incubate at 37 °C for 16 h or longer (*see* **Note 2**).
4. Dialyze against water. Alternatively, submit to gel filtration or reverse-phase HPLC to separate salts and peptides. Lyophilize peptides.
5. If it is necessary to convert the newly formed iminothiazolidinyl N-terminal residue to an alanyl group, proceed as follows: Dissolve the sample to, say, 0.5 mg/mL in deblocking buffer, (pH 7); add to Raney nickel (10-fold excess, [w/w] over polypeptide); and incubate at 50 °C for 7 h under an atmosphere of nitrogen. Cool and centrifuge briefly to pellet the Raney nickel. Store supernatant at −20 °C, or further analyze as required (s*ee* **Notes 5–8**).

4. Notes

1. The reactions described in **Subheading 3** are illustrated in **Fig. 1**. The method described is basically that used by Swenson and Fredrickson *(5)*, an adaptation of that of Jacobson et al. (*8*; *also see* **ref.** ***3***). The principal difference is that the earlier method ***(3,8)*** describes desalting (by gel filtration or dialysis) at the end of the modification step (*see* **Subheading 3**), followed by lyophilization and redissolution in a pH 9 buffer to achieve cleavage. Simple adjustment of pH as described in **Subheading 3.,** step 3 has the advantages of speed and avoiding the danger of sample loss upon desalting.

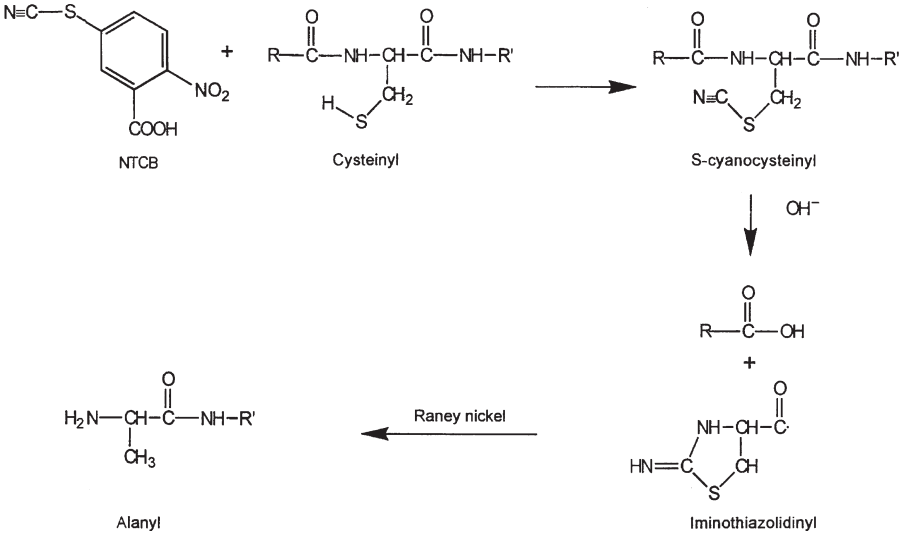

Fig. 1. Reactions in modification of, and cleavage at, cysteinyl residues by NTCB, and subsequent generation of alanyl N-terminal residue.

2. Swenson and Fredrickson *(5)* describe cleavage (**Subheading 3.,** step 3) at 37 °C for 6 h, but report yields of 60–80%. Other references recommend longer incubations of 12 h or 16 h at 37 °C to obtain better yields *(3,8,9)*.
3. A slightly modified procedure is described in *(9)*:
   a. 1 mg/mL sample in 20 m*M* borate buffer, pH 8.0, 6 *M* urea, mixed with NTCB, added as a 0.1 *M* solution in 33% (v/v) dimethylformamide, at the rate of 40 µL/mL sample solution. Incubation was at 25 °C for 1 h.
   b. Cleavage was by adjusting to pH 9.0 with NaOH and incubation at 55 °C for 3 h.
   c. Reaction was quenched by addition of 2-mercaptoethanol to an 80-fold excess over NCTB.
4. If the sample contains no intramolecular or intermolecular disulfide bonds, the DDT content of the modification buffer may be made less, at 1 m*M*. Note that nominally nonbonded cysteinyl residues may be involved in mixed disulfides with such molecules as glutathione or free cysteine.
5. If blockage of the N-terminal residue of the newly generated peptide(s) to the C-terminal side of the cleavage point(s) is not a problem (i.e., if sequencing is not required, e.g., **ref.** *5*), step 5 in **Subheading 3** may be omitted.

6. Reaction with Raney nickel (**Subheading 3.**, step 5) converts methionyl residues to (β-aminobutyryl residues.
7. Although Raney nickel is available commercially, Otieno ***(4)*** has reported that a more efficient catalyst may be obtained by the method he described, starting from Raney nickel– aluminum alloy. This is reacted with NaOH, washed, deionized, and washed again (under H_2 gas).
8. Treatment of protein with Raney nickel *without* prior treatment with NTCB causes desulfurization of methioninyl residues (to give (β-aminobutyryl residues) and of cysteinyl and cystinyl residues (to alanyl residues). Otieno ***(4)*** has suggested that this modification might be used to study dependence of protein function on Met and Cys content.

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Chemical Cleavage of Proteins at Asparaginyl-Glycyl Peptide Bonds

Bryan John Smith

1. Introduction

Reaction with hydroxylamine has been used to cleave DNA and to deacylate proteins (at neutral pH; *see* **Note 6**). At alkaline pH, however, hydroxylamine may be used to cleave the asparaginyl-glycyl bond (*see* **Note 1**). This cleavage tends to generate large peptides, as this pairing of relatively common residues is relatively uncommon, representing about 0.25% of amino acid pairs, according to Bornstein and Balian ***(1)***. It is therefore generally not useful for identification of proteins by mass mapping, which is better served by larger numbers of peptides. It may be a useful method for further cleavage of large peptides.

Cleavage at Asn-Gly bonds has been used for:

1. Generation of peptides for sequencing purposes (e.g., **ref. *2***).
2. Generation of peptides for use in mapping ligand binding sites (e.g. **ref. *3***) and phosphorylation sites (e.g. **ref. *4***).
3. Identification of sites of succinimide (cyclic amide) sites in proteins ***(5)***.
4. Cleavage of a fusion protein at the point of fusion of the constituent polypeptides (although it was noted that formation of hydroxamates may occur ***[6]***, as may minor cleavage reactions ***[3]***).

2. Materials

1. Cleavage buffer: 2*M* hydroxylamine.HCl, 2*M* guanidine.HCl, 0.2*M* KCO_3, pH 9.0.

Use Analar grade reagents and HPLC grade water (*see* **Note 2**). **Beware the mutagenic, toxic and irritant properties of hydroxylamine**. Wear protective clothing. Clear wet spillages with absorbent material or clear dry spillages with a shovel, and store material in containers prior to disposal.

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1. Stopping solution: Trifluoroacetic acid, 2% (v/v) in water (high-performance liquid chromatography [HPLC] grade).

3. Method

1. Dissolve the protein sample directly in the cleavage buffer, to give a concentration in the range 0.1–5 mg/mL. Alternatively, if the protein is in aqueous solution already, add 10 vol of the cleavage buffer (i.e., sufficient buffer to maintain pH 9.0 and high concentration of guanidine·HCl and hydroxylamine). Use a stoppered container (Eppendorf tube or similar) with small headspace, so that the sample does not dry out during the following incubation (*see* **Notes 3** and **4**).
2. Incubate the sample (in stoppered vial) at 45°C for 4 h.
3. To stop reaction, cool and acidify by addition of three volumes of stopping solution. Store frozen (−20°C) or analyze immediately (*see* **Note 5**).

4. Notes

1. The reaction involved in this cleavage is illustrated in **Fig. 1** and is described in more detail in **ref. *1***, with the proposed role of the succinimide being confirmed by Blodgett et al. ***(7)***. Note that the reaction of hydroxylamine is actually with the cyclic imide that derives from the Asn-Gly pair. The Asn-Gly bond itself is resistant to cleavage by hydroxylamine. Kwong and Harris ***(5)*** have reported cleavage at a presumed succinimide at an Asp-Gly bond. The succinimide occurs as an intermediate in the isomerization of aspartyl to *iso*-aspartyl, a reaction that involves the Gly residue. This can occur at sites that are in regions flexible enough to accommodate the three structures, so it is influenced by the neighboring sequence. This therefore affects cleavage efficiency. Bornstein and Balian ***(1)*** have reported an Asn-Gly cleavage yield of about 80% but lower efficiency may be experienced (as reported in **ref. *5***, for various conditions). Cleavage is to the carboxy-(C)-terminal side of the succinimide. The peptide generated to the C-terminal side is available for amino-(N)-terminal sequence analysis, for generation of internal sequence or to identify the site of the succinimide. The succinimide is stable enough to be found in proteins ***(5,8)***. Isomerization of the Asp to iso-Asp can affect immunogenicity and function (for instance, *see **[8]***). The *iso*-Asp may be detected by other assays (see ***[9]*** and references therein), or by termination of N-terminal sequencing, for both it and the succinimidyl residue are refractory to Edman sequencing chemistry. The succinimidyl version of a polypeptide is slightly more basic (by one net negative charge) than the aspartate version that forms after incubation in neutral pH ***(5)***. In addition to cleavage at Asn-Gly, treatment with hydroxylamine may generate other, lower yielding, cleavages. Thus, Bornstein and Balian ***(1)*** mention cleavage of Asn-Leu, Asn-Met, and Asn-Ala, while Hiller et al ***(3)*** report cleavage of Asn-Gln, Asp-Lys,

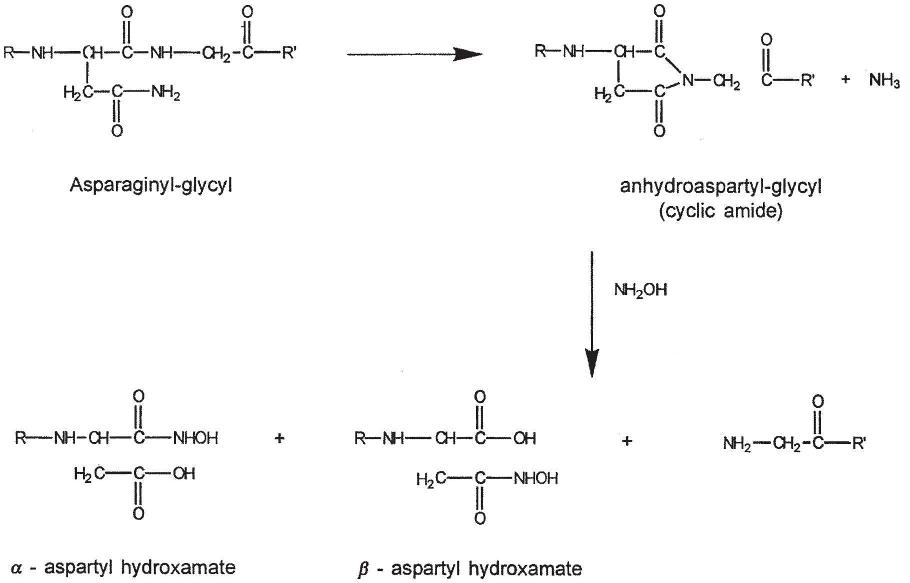

Fig. 1. Illustration of reactions leading to cleavage of Asn-Gly bonds by hydroxylamine.

Gln-Pro, and Asn-Asp. Prolonged reaction times tend to generate more of such cleavages. Treatment of protein with hydroxylamine may generate hydroxamates of asparagine and glutamine, these modifications producing more acidic variants of the protein *(6)*.

2. Inclusion of guanidine.HCl as a denaturant seems to be a factor in improving yields. Kwong and Harris *(5)* reported that omission of guanidine. HCl eliminated cleavage at an Asn-Gly site while allowing cleavage at an Asp-Gly site. The literature has other examples of the use of buffers lacking guanidineá·HCl, for instance, **refs.** ***3*** and ***5***. Both of these examples report use of a Tris-HCl buffer of approx pH 9.0, with **ref.** ***5*** including 1 m*M* EDTA and ethanol (10% v/v). Yet other examples ***(1,2,10)*** describe the use of more concentrated guanidine·HCl, at 6*M*, the cleavage buffer being prepared by titrating a solution of guanidine·HCl (6*M*, final) and hydroxylamine·HCl (2*M*, final) to pH 9.0 by addition of a solution of lithium hydroxide (4.5*M*). Note that preparation of this lithium hydroxide solution may generate insoluble carbonates that can be removed by filtration. Other reaction conditions were as described in **Subheading 3.**
3. As when making peptides by other cleavage methods, it may be advisable, prior to the above operations, to reduce disulfide bonds and alkylate cysteinyl residues (*see* Chapter 82). This denatures the substrate and prevents formation of interpeptide disulfide bonds. Niles and Christen ***(10)*** describe

alkylation and subsequent cleavage by hydroxylamine on scale of a few microliters.

4. The hydroxylamine cleavage method has been adapted to cleave proteins in polyacrylamide gel pieces ***(11)***. The cleavage buffer was 2 *M* hydroxylamine.HCl, 6 *M* guanidine.HCl, in 15 m*M* Tris titrated to pH 9.3 by addition of 4.5 *M* lithium hydroxide solution. Pieces of gel that had been washed in 5% methanol to remove sodium dodecyl sulfate (SDS), and then dried *in vacuo* were submerged in the cleavage solution (50–200 µL per 3 µL of gel) and incubated at 45°C for 3 h. Analysis by electrophoresis on a second SDS gel then followed. Peptides of about 10,000 Da or less tended to be lost during washing steps, and about 10% of sample remained bound to the treated gel piece. Recoveries in the second (analytical) SDS gel were reported to be approx 60% and cleavage yield was about 25%.
5. After the cleavage reaction has been stopped by acidification, the sample may be loaded directly onto reverse-phase HPLC or gel filtration for analysis/peptide preparation. Alternatives are dialysis and polyacrylamide gel electrophoresis (PAGE). The reaction itself may be stopped not by acidification, but by mixing with SDS-PAGE sample solvent and immediate electrophoresis ***(3)***.
6. In approximately neutral pH conditions, reaction of protein with hydroxylamine may cause esterolysis, and so may be a useful method in studying posttranslational modification of proteins. Thus, incubation in 1 *M* hydroxylamine, pH 7.0, 37°C for up to 4 h cleaved carboxylate ester-type ADP–ribose–protein bonds (on histones H2A and H2B) and arginine–ADP–ribose bonds (in histones H3 and H4) ***(12)***. Again, Weimbs and Stoffel ***(13)*** identified sites of fatty acid-acylated cysteine residues by reaction with 0.4 *M* hydroxylamine at pH 7.4, such that the fatty acids were released as hydroxamates. Omary and Trowbridge ***(14)*** adapted the method to release [^{3}H] palmitate from transferrin receptor in polyacrylamide gel pieces, soaking these for 2 h in 1 *M* hydroxylamine·HCl titrated to pH 6.6 by addition of sodium hydroxide.

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Enzymatic Digestion of Proteins in Solution and in SDS Polyacrylamide Gels

Kathryn L. Stone, Erol E. Gulcicek, and Kenneth R. Williams

1. Introduction

The completion of numerous genomes and the advancement of mass spectrometric analysis have enabled proteins of interest from complex biological mixtures to be identified at lower levels and considerably faster than using classical edman chemical sequencing. The approach is to enzymatically or chemically cleave the protein into smaller peptide fragments and analyze the digest / cleavage products using either high mass accuracy matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) ***(1)*** or tandem mass spectrometry (MS/MS) utilizing either MALDI or Electrospray ionization (ESI) sources ***(2)***. Following the mass spectral acquisition, proteins of interest are identified *in-silico* by matching the corresponding accurate peptide mass data (peptide mass fingerprinting) and the peptide fragment data (ion matching) to the theoretically digested and fragmented peptides in protein databases ***(3)***. For both of these types of mass spectrometric analyses, as well as for digests destined for edman chemical sequencing, it is important to obtain a high efficiency of digestion, with peptides in the 8 to 30 residue lengths preferable. Although several different approaches may be taken to cleave proteins, one of the most common is to digest the protein enzymatically with a relatively specific protease such as trypsin or lysyl endopeptidase. Since final purification is often dependent on SDS-PAGE, cleavage procedures that can either be carried out in the polyacrylamide gel matrix ***(4, 5)*** or that may be used on samples that have been blotted from SDS polyacrylamide gels onto PVDF ***(6)*** or nitrocellulose ***(6,7)*** membranes are extremely useful. The proteins are usually stained with Coomassie blue, or digestion compatible silver stains (used with polyacrylamide gels) prior to excision and proteolytic digestion. The resulting peptides are separated

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by reverse-phase HPLC or UPLC (ultra high pressure or ultra performance liquid chromatography), and in most instances, the LC system is directly attached to a mass spectrometer for real time protein identification analysis. Relatively straightforward solution and in-gel digestion procedures, including robotic digestion, that have been used extensively in the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University will be described in this chapter, whereas a procedure suitable for in situ digestion of SDS-PAGE blotted proteins is described in Chapter 101.

2. Materials

2.1. Enzymatic Digestion of Proteins

1. Enzymatic digestion of proteins is usually accomplished using either sequencing grade, modified trypsin (from Promega, [Madison, WI] or Boehringer Mannheim, [Indianapolis, IN]) or lysyl endopeptidase (Achromobacter Protease I from Achromobacter lyticus) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Occasionally, chymotrypsin or endoproteinase Glu-C from (Boehringer Mannheim) may also be used. All enzyme stocks are divided into 100 µL aliquots and stored at −20 °C.
2. Sequencing-grade chymotrypsin and modified trypsin (Boehringer Mannheim): prepare by dissolving the dried manufacturer supplied 100-µg aliquots in 1 mL 1 m*M* HCl to make a 0.1 mg/mL stock solution that appears to be stable for at least 6 months at −20 °C.
3. Sequencing grade, modified trypsin (Promega): dissolve the manufacturer supplied 20-µg aliquot in 200 µL 1 m*M* HCl to make a 0.1 mg/mL stock solution that appears to be stable for at least 6 months at −20 °C.
4. Endoproteinase Glu-C: dissolve the 50-µg aliquot from the manufacturer in 500 µL 50 m*M* NH_4HCO_3. According to the manufacturer, the dissolved enzyme is stable for 1 month at −20 °C.
5. Lysyl endopeptidase: dissolve 2.2 mg as purchased in 2.2 mL of 2 m*M* Tris-HCl, pH 8.0, to make a 1 mg/mL stock, which according to the manufacturer is stable for at least 2 years when stored at −20 °C. More dilute solutions are made by adding 10 µL of this 1 mg/mL stock solution to 90 µL 10mM NH_4HCO_3, pH 8.0, for a 0.1 mg/mL stock.
6. Pepsin (Sigma Chemical Co., St. Louis, MO): dissolve in 5% formic acid (Mallinckrodt-Baker, Phillipsburg, NJ) at a concentration of 0.1 mg/mL.
7. Digestion buffer for in-solution digestion: 8 *M* urea, 0.4 *M* NH_4HCO_3. Prepare by dissolving 4.8 g Pierce Sequanal-Grade urea and 0.316 g Mallinckrodt-Baker ammonium bicarbonate in H_2O to make a final volume of 10 mL.
8. 50% CH_3CN/water: : add 5mL 100% CH_3CN to 5 mL H_2O.
9. 50% CH_3CN/50mM NH_4HCO_3: dissolve 0.0395 g in 5 mL H_2O plus 5mL 100% CH_3CN
10. 50% CH_3CN/10mM NH_4HCO_3: dissolve 0.0079 g in 5 mL H_2O plus 5mL 100% CH_3CN

11. In gel digestion buffer 10mM NH_4HCO_3 (manual digestion): dissolve 0.0079 g in 10 mL H_2O
12. 5mM NH_4HCO_3 (robotic digestion): dissolve 0.0079 g in 20 mL H_2O.
13. 45 m*M* DTT, Pierce Chemical Co. (Rockford, IL) solution for protein reduction: dissolve 69 mg DTT in 10 mL H_2O.
14. 100 m*M* iodoacetic acid (IAA), Pierce Chemical Co. (Rockford, IL) solution for alkylation: dissolve 185.9 mg IAA in 10 mL H_2O.
15. 200 m*M* methyl 4-nitrobenzene sulfonate, Aldrich Chemical Co. (St. Louis, MO) solution for cysteine modification: is made by dissolving 0.0434 g methyl 4-nitrobenzene sulfonate in 100% CH_3CN. This solution is made immediately before use and is not stored.
16. 0.1% TFA/60% CH_3CN solution for peptide extraction from the gel slices: add 50 µL 100% trifluoracetic acid (TFA) to 20 mL H_2O and 30 mL 100% CH_3CN.
17. For dissolving dried digest: 70% formic acid: prepare from Pierce 99+% formic acid by mixing 70 µL 99% formic acid with 30 µL H_2O.
18. 0.1% TFA for sample dilution prior to HPLC or MS analysis: add 50 µL 100% trifluoracetic acid (TFA) to 50 mL H_2O.

3. Methods

3.1. Digestion of Proteins in Solution

3.1.1. Sample Preparation for in Solution Digestion

Proper sample preparation for proteins is critical both in avoiding sample loss and in ensuring successful digestion (*see* **Note 1**). If the sample contains a sufficiently low level of salt and glycerol (such that the concentration of salt and glycerol in the final digest will be less than the equivalent of 1 *M* NaCl and 15% glycerol, respectively), it may simply be reduced to dryness in a SpeedVac prior to carrying out the digest in the tube in which it was dried. Samples containing higher levels of salts, glycerol, and/or detergents, such as SDS, may be precipitated using either trichloroacetic acid (TCA) or acetone in order to remove the salts, glycerol, and detergents. To TCA-precipitate the protein, 1/9th volume of 100% TCA is added to the sample prior to incubating on ice for 30 min. The sample is then centrifuged (10,000g/15 min) and the supernatant is removed. Residual TCA is then removed by suspending the pellet in 50 µL cold acetone. After vortexing and centrifuging, the supernatant is removed with a pipet. The air-dried pellet is then digested as described below. To acetone precipitate the protein, it is often necessary first to reduce the salt concentration by dialysis vs 0.05% SDS, 5 m*M* NH_4HCO_3. At this point, the volume is reduced to <50 µL in a SpeedVac, and 9 volumes of cold acetone are added followed by a 1 h incubation at −20 °C. The sample is then centrifuged and the pellet washed (as described in **Subheading 3.1**) with 50 µL cold acetone. When large amounts of SDS are present, the acetone wash should be repeated once or twice to remove excess detergent. The recommended minimum protein concentration during either precipitation is 100 µg/mL, and for TCA precipitation, the glycerol concentration should be below 15%.

Newer digestion additives such as RapiGest™ (Waters Corp., Milford, MA) and PPS Silent™ (Protein Discovery, Inc., Knoxville, TN) surfactants aid in denaturing and solubilizing proteins. For either denaturant, a volume equal to the protein solution volume is added for a 1:1 ratio.

3.1.2. Digestion of Proteins in Solution with Trypsin, Lysyl Endopeptidase, Chymotrypsin, and Endoproteinase GLUC

After the salts and detergents have been removed/minimized, the sample is ready for digestion. Typically, trypsin is the enzyme of choice because of its relatively high cleavage specificity (the COOH-terminal side of lysine and arginine) and its ability to digest insoluble substrates. That is, proteins that are only partially soluble in the digest buffer will often cleave with trypsin as evidenced by rapid clearing of the solution. Cleavage occurs more slowly when there is an acidic residue following the lysine or arginine and not at all when a lysine-proline or arginine-proline linkage is present. Lysyl endopeptidase only cleaves on the COOH-terminal side of lysine. As a result, it provides longer peptides than trypsin. Based on the average occurrence of lysine (5.7%) and arginine (5.4%) in proteins in the Protein Identification Resource Database (as of 2001), the average length of a tryptic and lysyl endopeptidase peptide is about 9 and 18 residues, respectively. However, since lysyl endopeptidase does not generally cleave insoluble substrates and since we have occasionally encountered proteins that will digest with trypsin, but not with lysyl endopeptidase, we generally use trypsin. Occasionally, if there is sufficient protein to carry out two separate digests (if needed to increase protein coverage), we would initially use lysyl endopeptidase, and then do a second digest using trypsin.

Chymotrypsin is occasionally used to redigest larger peptides or proteins that fail to digest with trypsin. It readily cleaves on the COOH-terminal side of tryptophan, tyrosine, and phenylalanine, and generally gives partial cleavage after leucine, methionine, and several other amino acids. However, its specificity is too broad to be of general use. Endoproteinase Glu-C is relatively specific in that it cleaves after glutamic acid in either ammonium bicarbonate (pH 8.0) or ammonium acetate (pH 4.0) buffers and after both aspartic acid and glutamic acid in phosphate buffers (pH 7.8). It also does not generally cleave insoluble substrates, and the HPLC profiles we have obtained with this enzyme usually suggest relatively incomplete cleavage with the resulting generation of overlapping peptides in lower yield than might often be obtained with trypsin.

Although this brief survey of proteolytic enzymes is far from complete, it does cover most of the enzymes that are frequently used. The enzymatic digestion protocol outlined below may be used with any of these four enzymes, providing the appropriate buffer changes are made in the case of Glu-C.

1. Dissolve the dried or precipitated protein in 20 µL 8 *M* urea, 0.4 *M* NH_4HCO_3. At this point, an aliquot can be removed for acid hydrolysis/ion-exchange amino acid analysis if > 1 µg of sample is expected (*see* **Note 2**). If using the RapiGest or PPS Silent Surfactant, dissolve the dried pellet in 20 µl of 0.2%. Add an equal volume of water for a final concentration of 0.1% denaturant.
2. Check the pH of the sample by spotting 1–2 µL on pH paper. If necessary, adjust the pH to between 7.5 and 8.5.
3. Add 5 µL 45 m*M* DTT and incubate at 50 °C for 15 min to reduce the protein (*See* **Note 3**).
4. After cooling to room temperature, alkylate the protein by adding 5 µL 100 m*M* IAA and incubating at room temperature for 15 min (*see* **Note 3**).
5. For samples in urea, dilute the digestion buffer with H_2O so the final digestion will be carried out in 2 *M* urea, 0.1 *M* NH_4HCO_3.
6. Add the enzyme in a 1/25, enzyme/protein (wt/wt) ratio (*see* **Note 4**).
7. Incubate at 37 °C for 16–24 h.
8. Stop the digest by acidifying the sample with TFA for injection onto a reverse-phase HPLC system or by freezing it.

3.1.3. Digestion of Proteins in Solution with Pepsin

Although the very broad specificity of pepsin hinders its routine use for comparative peptide mapping, its low optimum pH performance enables it to cleave proteins that might otherwise be intransigent. It is also applicable for digesting relatively small peptides and, particularly, for studies directed at identifying disulfide bonds (*see* **Note 5**). Although pepsin cleaves preferentially between adjacent aromatic or leucine residues, it has been shown to cleave at either the NH_2- or COOH- terminal side of any amino acid, except proline. A typical digestion procedure is as follows:

1. Dissolve the dried protein in 100 µL 5% formic acid.
2. Add pepsin at a 1:50, enzyme:protein ratio(wt:wt).
3. Incubate the sample at room temperature for 1–24 h. with the time of incubation being dependent on the desired extent of digestion.
4. Dry the digest in a SpeedVac prior to dissolving in 0.05% TFA and immediately inject onto a reverse-phase HPLC system.

3.2. Digestion of Proteins in SDS Polyacrylamide Gels

3.2.1. Sample Preparation for in Gel Digestion

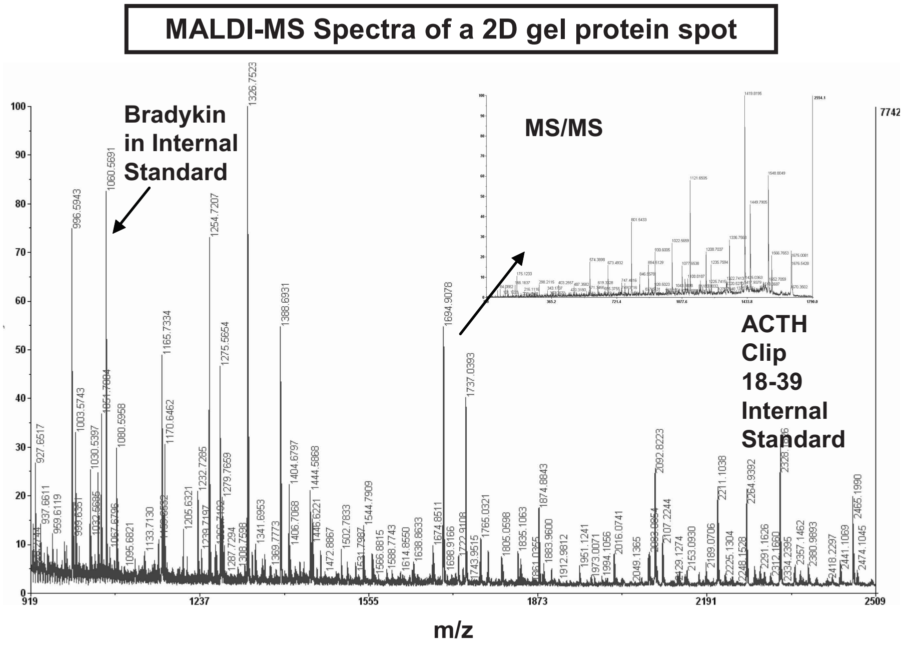
As in the case of samples destined for in-solution digests, care must also be exercised in preparing samples for SDS-PAGE so that sample losses are minimized (*see* **Note 6**) and so that the final ratio of protein/gel matrix is as high as possible (*see* **Note 7**). Although prior carboxymethylation does not appear to be essential with in-gel digests, the presence of greater than 10–20% carbohydrate (by weight) often appears to hinder cleavage significantly (*see* **Note 3**). Samples that have been purified by SDS-PAGE can be digested directly in the gel matrix, thereby eliminating the need for electroelution or electroblotting of the intact proteins from the gel.

SDS PAGE-separated proteins destined for in-gel digestion can be stained in a variety of ways, including using commercially available staining kits. The sensitivity of the stains vary with the Coomassie blue R250 being the least sensitive. Several of the more common stains are 1) classical Coomassie blue R250 staining using 0.1% Coomassie blue in 50% methanol, 10% acetic acid for 1 h. prior to destaining with 50% methanol, 10% acetic acid for a minimum of 2 h. (*see* **Note 8**). 2) Colloidal Blue staining such as with the GelCode® Blue Stain Reagent from Pierce 3) digestion compatible silver staining which can be done using Invitrogen's SilverQuest™ kit and 4) UV-based SYPRO® Ruby Protein Gel Stain (Molecular Probes™). Alternatively, staining the proteins of interest can be avoided when sufficient sample is available (*see* **Note 9**) and the band of interest is well separated. This can be done with stained Molecular Weight markers in an additional guide lane, or running the sample of interest in 2 lanes and staining the 1 lane. The protein of interest can then be excised from the gel using this guide. However, in this instance, the gel still must be exposed to 50% methanol, 10% acetic acid for 3 h. to ensure adequate removal of SDS prior to excising the protein band of interest.

The % polyacrylamide gel that is used is determined by the size of the protein. Proteins >100 kDa are typically electrophoresed in 7–10% polyacrylamide gels, whereas smaller proteins are electrophoresed in 10–17.5% polyacrylamide. The in gel digestion procedure is suitable for both 1 and 2 dimensional gels with the latter digests often performed on a digester robot. The protein of interest is excised using a razor blade and tweezers, and the gel spots are then placed in an eppendrof tube that has been pre-washed with 0.1% TFA/ 60% CH_3CN (to minimize contaminants). Alternatively, a robotic gel picker such as the GE Healthcare Ettan Spot Picker can be used to excise the proteins of interest. This spot picker cuts out a 2mm disc and deposits the gel piece in a 96 well plate. Care must always be taken to avoid keratin contamination which can confound any protein identification analysis. **Figure 1** contains a typical MALDI-Tof/Tof spectra of an in gel tryptic digest.

3.2.2. In-Gel Digestion of Proteins with Trypsin and Lysyl Endopeptidase

1. Determine the approximate volume of gel to be digested (length × width × thickness).
2. Cut the gel band(s) containing the protein of interest into approx 1 × 2 mm pieces, and place them in an Eppendorf tube or a 96 well plate for robotic digestion. Repeat for the "blank" section of gel.



MALDI-MS Spectra of a 2D gel protein spot

Fig. 1. In-gel trypsin digestion of an unknown protein spot from a 2D gel. Human plasma was analyzed by 2D gel electrophoresis following IgY12 depletion (Beckman-Coulter) and Cy dye labeling as per the GE Healthcare Differential (fluorescence) gel electrophoresis protocol. Protein spots of interest were determined using GE Healthcare DeCyder software of the fluorescent gel image, and excised robotically (GE Healthcare Ettan Spot Picker) as described in **Subheading 3.** 100% of the digest was loaded onto an AB 4800 MALDI-Tof/Tof plate after dissolving in 0.6nl of the alpha-cyano-4-hydroxy cinnamic acid matrix containing 1 fmol of bradykinin and 2 fmols of ACTH clip as internal standards. The sample was analyzed using both peptide mass fingerprint and MS/MS of 10 peptides. The inset panel shows the MS/MS spectra obtained from the mass at 1694.9. A Mascot (Matrix Science) database search identified this sample as Vitamin D-binding protein precursor. The estimated amount of sample analyzed is 0.5 to 1 fmol based on the bradykinin internal standard.

3. Add 250 µL (or more, if necessary to cover the gel pieces) 50% CH_3CN/50% water to the gel pieces.
4. Wash for 5 min at room temperature on a rocker table.
5. Remove wash.
6. Repeat steps 3–5 using 250 µl 50% CH_3CN/50mM NH_4HCO_3
7. Repeat steps 3–5 using 250 µl 50% CH_3CN/10mM NH_4HCO_3
8. Dry the washed gel pieces in a SpeedVac
9. Make up the enzyme solution by diluting 5 µL 0.1 mg/mL enzyme stock solution with 10 µL 10mM NH_4HCO_3 for every 15 mm^3 gel that is to be digested.

10. Rehydrate gel pieces with the enzyme solution from **step 9**, which should be equal in volume to that of the gel pieces and should provide a final enzyme ratio of about 0.5 μg/15-mm^3 gel volume.
11. If the gel pieces are not totally immersed in the enzyme solution, add an additional volume of 10mM NH_4HCO_3 to submerge the gel pieces totally.
12. Incubate at 37 °C for 5 to 16 h.
13. After incubation, the supernatant can be removed and injected on to a HPLC column or LC-MS/MS system. Gels pieces can also be further extracted as in steps 19–20 and Cysteine modification, if desired, can be performed as follows in steps 14 – 18
14. Estimate the total volume of the sample plus gel.
15. Calculate the volume of 45 m*M* DTT needed to give a final (DTT) of about 1 m*M* in the sample.
16. Add the above volume of 45 m*M* DTT and then incubate at 50 °C for 20 min.
17. Remove samples from the incubator; cool to room temperature and add an equal volume of 100 m*M* IAA or 200 m*M* methyl 4-nitrobenzene sulfonate (MNS) (*see* **Note 10**) as the volume of DTT added in step 16.
18. For IAA alkylation, incubate at room temperature in the dark for 20 min; for MNS treatment, incubate at 37 °C for 40 min.
19. Extract peptides by adding at least 100 μL 0.1% TFA, 60% CH_3CN (or, if it is greater, a volume equal to the gel volume estimated in step 2), and shake on a rocker table at room temperature for at least 40 min.
20. Sonicate for 5 min in a water bath sonicator.
21. Remove and save the supernatant, which contains the released peptides, and repeat steps 19 and 20.
22. SpeedVac dry the combined washes from step 21.
23. Redissolve the dried samples in 3 to 5 μL 70% formic acid (to dissolve the peptides) and bring to proper injection volume using 0.1% TFA.
24. The sample is now ready for reverse-phase HPLC peptide separation as described in Chapter 102 or mass spectrometric analysis.

3.2.3. In-Gel Robotic Digestion of Proteins with Trypsin

A digester robot such as the GE Healthcare Ettan Ta digester is required for robotic digestion.

1. Excise the protein of interest by hand or using a gel picker (e.g. Ettan spot picker) and place in an 96 well plate (*see* **Note 11)**.
3. Add 50 μL 50% CH_3CN/50% water to the gel pieces.
4. Wash for 3 min at room temperature.
5. Remove wash.
6. Repeat steps 3–5.
7. Repeat steps 3–5 using 50 μl 5mM NH_4HCO_3.
8. Air dry gel pieces for 5 min.
9. Make up the enzyme solution by diluting 100 μL 0.1 mg/mL enzyme stock solution with 2.9 mL 5mM NH_4HCO_3.
10. Rehydrate gel pieces with 15 μl of the enzyme solution from step 9.

11. Incubate at 37 °C for 5 to 16 h.
12. After incubation, the supernatant can be removed and injected on to a HPLC column or LC-MS/MS system. For MALDI-MS analysis, speedvac the digest to dryness and re-dry two times from 20 µl water. The sample is now ready to be dissolved in matrix and analyzed.

4. Notes

1. In many instances, large losses occur during the final purification steps when the protein concentrations are invariably lower. Hence, although ultrafiltration or dialysis of a 5 mg/mL crude solution of a partially purified enzyme may lead to nearly 100% recovery of activity, similar treatment of a 25 µg/mL solution of the purified protein might well lead to significant, if not total loss of activity owing to nonspecific adsorption. Similarly, the effectiveness of organic and acid-precipitation procedures often decreases substantially as the final protein concentration is decreased below about 100 µg/mL. Whenever possible, therefore, the final purification step should be arranged such that the resulting protein solution is as concentrated as possible and, ideally, can simply be dried in a SpeedVac prior to enzymatic digestion. In this regard, it should be noted that a final NaCl concentration of 1 *M* does not significantly affect the extent of trypsin digestion ***(8)***. When it is necessary to carry out an organic or acid precipitation to remove salts or detergents, the protein should first be dried in a SpeedVac (in the 1.5-mL tube in which it will ultimately be digested) prior to redissolving or suspending in a minimum volume of water and then adding the acetone or TCA. In this way, the protein concentration will be as high as possible during the precipitation and losses will be minimized. Two common contaminants that are extremely deleterious to enzymatic cleavage are detergents (as little as 0.005% SDS will noticeably decrease the rate of tryptic digests carried out in the presence of 2 *M* urea ***[8]***) and ampholines. Since detergent removal is often associated with protein precipitation, and since many detergents (such as SDS) form large micelles, which cannot be effectively dialyzed, it is usually preferable to extract the detergent from the protein (that has been dried in the tube in which it will be digested) rather than to dialyze it away from the protein. In the case of ampholines, our experience is that even prolonged dialysis extending over several days with a 15,000-Dalton cutoff membrane is not sufficient to decrease the ampholine concentration to a level that permits efficient trypsin digestion. Rather, the only effective methods we have found for complete removal of ampholines are TCA precipitation, hydrophobic or reverse-phase chromatography, or SDS-PAGE followed by staining and destaining.

2. It is particularly important to quantify samples using amino acid analysis if protein profiling (i.e. comparative analysis) is to be done. For samples in solution, the aliquot for amino acid analysis should be taken either immediately prior to drying the sample in the tube in which it will be digested or after redissolving the sample in 8 *M* urea, 0.4 *M* NH_4HCO_3. Although up to 10 µL of 8 *M* urea is compatible with acid hydrolysis/ion-exchange amino acid analysis, this amount of urea may not be well tolerated by PTC amino acid analysis. Hence, in the latter case, the amino acid analysis could be carried out prior to drying and redissolving the sample in urea. Samples destined for comparative 2D gel analysis such as DIGE should also be quantified using amino acid analysis prior to running the 2D gel. The aliquot for analysis can be taken after the samples have been dissolved in the gel loading buffer. By having an accurate protein concentration, comparative protein profiling experiments are themselves more accurate.
3. Since many native proteins are resistant to enzymatic cleavage, it is usually best to denature the protein prior to digestion. Although some proteins may be irreversibly denatured by heating in 8 *M* urea, this treatment is not sufficient to denature transferrin. In this instance, prior carboxymethylation, which irreversibly modifies cysteine residues, brings about a marked improvement in the resulting tryptic peptide map ***(8)***. Another advantage of carboxymethylating the protein is that this procedure enables cysteine residues to be identified during edman amino acid sequencing. Cysteine residues have to be modified in some manner prior to edman sequencing to enable their unambiguous identification. Under the conditions that are described in **Subheading 3.**, the excess dithiothreitol and iodoacetic acid do not interfere with subsequent digestion. Although carboxymethylated proteins are usually relatively insoluble, the 2 *M* urea that is present throughout the digestion is frequently sufficient to maintain their solubility. However, even in those instances where the carboxymethylated protein precipitates following dilution of the 8 *M* urea to 2 *M*, trypsin and chymotrypsin will usually still provide complete digestion. Often, the latter is evidenced by clearing of the solution within a few minutes of adding the enzyme. Alternatively, the newer denaturants RapiGest™ and PPS Silent™ Surfactant can be used to try to better solubilize the proteins of interest. If carboxymethylation is insufficient to bring about complete denaturation of the substrate, an alternative approach is to cleave the substrate with cyanogen bromide (1000-fold molar excess over methionine, 24 h at room temperature in 70% formic acid). The resulting peptides can then either be separated by SDS-PAGE (since they usually do not separate well by reverse-phase HPLC) or, preferably, they can be enzymatically digested with trypsin or lysyl endopeptidase and then separated by reverse phase HPLC or LC-MS/MS.

If this approach fails, the protein may be digested with pepsin, which, as described above, is carried out under very acidic conditions or can be subjected to partial acid cleavage ***(9)***. However, the disadvantage of these latter two approaches is that they produce an extremely complex mixture of overlapping peptides. Finally, extensive glycosylation (i.e., typically >10–20% by weight) can also hinder enzymatic cleavage. In these instances, it is usually best to remove the carbohydrate prior to beginning the digest. In the case of in gel digests, this may often be best carried out immediately prior to SDS-PAGE, which thus prevents loss (owing to insolubility) of the deglycosylated protein and effectively removes the added glycosidases.

4. Every effort should be made to be used as a high substrate and enzyme concentration as possible to maximize the extent of cleavage. Although the traditional 1:25, weight:weight ratio of enzyme to substrate provides excellent results with milligram amounts of protein, it will often fail to provide complete digestion with low nanogram amounts of protein. For instance, using the procedures outlined above, this weight:weight ratio is insufficient to provide complete digestion when the substrate concentration falls below about 20 μg/mL ***(8)***. The only reasonable alternative to purifying additional protein is either to decrease the final digestion volume or to compensate for the low substrate concentration by increasing the enzyme concentration. The only danger in doing this, of course, is the increasing risk that some peptides may be isolated that are autolysis products of the enzyme. Assuming that only enzymes, such as trypsin, chymotrypsin, lysyl endopeptidase, and Protease V8 are used, whose sequences are known, it is usually better to risk sequencing a peptide obtained from the enzyme (which can be quickly identified via a data base search) than it is to risk incomplete digestion of the substrate. Often, protease autolysis products can be identified by comparative HPLC peptide mapping of an enzyme (i.e., no substrate) control that has been incubated in the same manner as the sample and by subjecting candidate HPLC peptide peaks to matrix-assisted laser desorption mass spectrometry prior to edman sequencing. The latter can be extremely beneficial both in identifying (via their mass) expected protease autolysis products and in ascertaining the purity of candidate peptide peaks prior to edman sequencing. For sequencing by LC-MS/MS analysis, it is not as critical to eliminate the trypsin/enzyme autolysis products since a database search will identify these peptides. To promote more extensive digestion, we have sometimes used enzyme:substrate mole ratios that approach unity.
5. One approach to identifying disulfide-linked peptides is to comparatively HPLC peptide map a digest that has been reduced/carboxymethylated vs one that has only been carboxymethylated (thus leaving disulfide-linked

peptides intact). In this instance, pepsin offers an advantage in that the digest can be carried out under acidic conditions where disulfide interchange is less likely to occur.

6. As in the case of in-solution digests, care must be exercised to guard against sample loss during final purification. Whenever possible, SDS (0.05%) should simply be added to the sample prior to drying in a SpeedVac and subjecting to SDS-PAGE. Oftentimes, however, if the latter procedure is followed, the final salt concentration in the sample will be too high (i.e., >1 *M*) to enable it to be directly subjected to SDS-PAGE. In this instance, the sample may either be concentrated in a SpeedVac and then precipitated with TCA (as described above) or it may first be dialyzed to lower the salt concentration. If dialysis is required, the dialysis tubing should be rinsed with 0.05% SDS prior to adding the sample, which should also be made 0.05% in SDS. After dialysis versus a few micromolar NH_4HCO_3 containing 0.05% SDS, the sample may be concentrated in a SpeedVac and then subjected to SDS PAGE (note that samples destined for SDS PAGE may contain several % SDS). Another approach that works extremely well is to use a SDS polyacrylamide gel containing a funnel-shaped well that allows samples to be loaded in volumes as large as 300 µL ***(10)***.
7. In general, the sample should be run in as few SDS-PAGE lanes as possible to maximize the substrate concentration and to minimize the total gel volume present during the digest. Whenever possible, a 0.5–0.75-mm thick gel should be used. In general, we recommend using 0.5 µg enzyme/15 mm^3 of gel with the only caveat being that we use a corresponding lower amount of enzyme if the mole ratio of protease/substrate protein would exceed unity.
8. Since high concentrations of Coomassie blue interfere with digestion, it is best to use the lowest Coomassie blue concentration possible and to stain for the minimum time necessary to visualize the bands of interest. In addition, the gel should be well destained so that the background is close to clear.
9. Although most estimates of protein amounts are based on relative staining intensities, our data suggest there is a 5–10-fold range in the relative staining intensity of different proteins. Obviously, when working in the low pmol/fmol levels, such a 5–10-fold range could well mean the difference between success and failure.
10. For edman sequencing, modification of cysteine residues with MNS provides the advantage that the resulting S-methyl cysteine phenylthiohydantoin derivative elutes in a favorable position (i.e., between PTH-Tyr and PTH-Pro) using an Applied Biosystems PTH C-18 column.
11. Due to the manner in which the robot operates, it is necessary to not digest too large a gel volume. Recommended gel volumes are 2mm discs.

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On-PVDF Protein Digestions for N-terminal Sequencing and Peptide Mass Fingerprinting

Victoria Pham, William Henzel, and Jennie R. Lill

1. Introduction

Two common analytical techniques employed for the characterization of proteins are N-terminal sequencing and peptide mass fingerprinting (PMF). ***(1)*** N-terminal sequencing employing the chemistries first described by Pehr Edman ***(2)*** is routinely used in many laboratories for deciphering if correct translational and post-translational processing of a protein has occurred. Peptide mass fingerprinting on the other hand, is used to determine if the correct protein has been cloned and translated, to look for mutations within a known protein sequence, and to look for posttranslational modifications.

A typical format for the N-terminal sequencing of protein is to transfer the sample onto a PVDF membrane. This is achieved by first separating proteins by SDS-PAGE, followed by electro-blotting onto PVDF membrane where they remain immobilized and stable until analysis. Edman chemistries require a free α-amino group at the N-terminus of the protein in order for phenylisothiocyante (PITC) to couple with the amino group of the terminus. However, many proteins expressed in mammalian cell lines are N-terminally blocked with co- or post translational modifications such as pyroglutamyl, acetyl or formyl groups, which render Edman chemistry inaccessible. Several protocols are available to remove these N-terminal modifications for example (deacetylation and pyroglutamyl). However, in some cases, e.g. for proteins with a pyroglutamyl group followed by a proline residue, removal of the modified group is not possible due to hindered stereochemistry. Under such situations, PVDF-imbedded N-terminally blocked proteins can be identified from their internal fragments generated from on-PVDF chemical or proteolytic cleavage.

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Peptide mass fingerprinting (PMF) involves the enzymatic or chemical proteolytic digestion of a protein into constituent peptides. Each protein will produce a unique signature of resultant peptides that can be compared against a list of theoretical masses to identify the protein, and also to look for alterations in sequence such as mutations and post translational modifications ***(3)***.

For characterization purposes, depending on the availability of the protein and the information that one needs to obtain, both Edman degradation and PMF analysis can be utilized since the 2 techniques are complimentary with each other. Here we describe protocols for the rapid on-PVDF enzymatic cleavage of proteins which can subsequently be analyzed either by Edman degradation or by PMF.

2. Materials

2.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Running buffer (10X): 247 m*M* Tris, 1.92 *M* glycine, 34.7 m*M* SDS, store at room temperature (RT).
2. Precast 4-20% Tris-glycine (Invitrogen, Carlsbad, CA).
3. Sample buffer (2X): 0.5 *M* Tris-HCl, pH 8.0, 20% SDS, 0.5% bromophenol blue, 26 % glycerol (in-house), store at RT.
4. Dithiothreitol DTT (BioVectra, Oxford, CT).
5. N-isopropyl iodoacetamide (NIPIA) (synthesized in-house).

2.2. Electroblotting

1. CAPS buffer: 10 m*M*, pH 11.0 containing 10 m*M* thioglycolic acid and 20% methanol, store at RT.
2. Problott PVDF membrane (Applied Biosystems, Foster City, CA).
3. Methanol at HPLC-grade (Burdick & Jackson, Muskegon, MI).

2.3. Staining/Destaining of PVDF Membrane

1. Staining solution: 0.1% Coomasie brilliant blue R250 in 50% methanol/10% acetic acid (in-house), store at RT.
2. Destaining solution: 50% methanol/10% acetic acid/MQ water (in-house), store at RT.

2.4. On-Membrane Chemical Cleavage for Edman Degradation

1. Mouse antihuman OX40 ligand antibody (R & D Systems, Minneapolis, MN).
2. Asp/Pro cleavage solution: 10% acetic acid containing 7 *M* guanidine, pH 2.5.

2.5. Rapid on-Membrane Digestion for Automated Edman Degradation

1. Human IL-20 receptor alpha poly his (purified in-house).
2. Clostripain (Calbiochem, San Diego, CA), trypsin (Promega, Madison, WI) store at −80 °C.
3. Zwittergent 3–16 (Calbiochem, San Diego, CA).
4. 0.1 *M* Tris-HCl, pH 8.0, 2 m*M* DTT, 1 m*M* $CaCl_2$.

2.6. On-Membrane Digestion for Peptide Mass Fingerprinting (PMF)

1. Recombinant human growth hormone (rec-HGH) (purified in-house), store at –80 °C.
2. Matrix α-cyano-4-hydroxycinnaminic acid (CHCA): nitrocellulose (NC) (20:5 w/w) in acetone: isopropyl alcohol at 1:1 ratio.
3. α-Cyano-4-hydroxycinnamic Acid (CHCA) (Sigma, St Louis, MO), NC (BioRad, Hercules, CA).
4. Acetone, isopropyl alcohol, acetonitrile at HPLC-grade (Burdick & Jackson, Muskegon, MI).

2.7. Instrumentation

1. Procise N-terminal sequencer, model 494 (Applied Biosystems, Foster City, CA).
2. Voyager-DE-STR MALDI-TOF (Applied Biosystems, Foster City, CA).

3. Methods

On-PVDF chemical cleavage has been widely used without pretreatment of the membrane. However, when on-PVDF enzymatic digestion is performed, the unoccupied sites on the membrane must be blocked. If these sites are not blocked enzyme activity maybe hindered, and also the ratio of enzyme to analyte upon analysis will be overwhelming. PVP-360 has previously been reported as a blocking agent for on-membrane digestion ***(4)*** and more recently we reported the use of Zwittergent 3–16, a sulfobetaine type detergent, with a hydrophobic un-branched 16-C chain, which binds strongly to the PVDF membrane, as an alternative surfactant ***(3)***. Our results indicated that Zwittergent 3–16 is a better blocking agent than PVP-360, and as a consequence more efficient on-PVDF digestion for N-terminal sequencing or PMF was observed.

3.1. SDS-PAGE and Electroblotting

1. Proteins are reduced with DTT (10 m*M* in 2X sample buffer) at 95 °C for 10 min and alkylated with NIPIA at a final concentration of 20 mM at RT for 20 min.
2. Proteins are then separated on a precast 4–20% Tris-glycine gel for 120 min at 120 V.

3.2. Electroblotting

1. Following separation, equilibrate gel in CAPS buffer (*see* **Note 1**) at RT for 10 min.
2. Electro-blot gel onto a prewetted PVDF membrane in CAPS buffer for 45-60 min at 250 mA (*see* **Note 2**)

3.3. Staining & destaining

1. Visualize proteins by staining in coomasie blue R250 staining solution for 30–60 s.
2. Destain PVDF membrane with 50% MeOH/ 10% acetic acid/H_2O for 2 min and extensively rinse with MQ H_2O (10 × 1.0 mL).
3. Store membrane at RT until further analysis.

3.4. On-PVDF Chemical Cleavage for Edman Degradation

1. Excise the band of interest and wet with 1–2 µL of methanol, rinsed with MQ water (10 × 1.0 mL).
2. Add 30 µL of diluted acid **(2.4)** and incubate in a thermocycler tube at 90 °C for 1 h (*se*e **Note 3**)
3. After incubation, wash the protein band with MQ water (10 × 1.0 mL) and dry in the Speed Vac.
4. Peptides can then be analyzed by N-terminal sequencing to identify the protein (*see* **Note 4**).

3.5. Rapid on-Membrane Digestion for Automated Edman Degradation

1. Protein bands are wetted with 1-2 µL of methanol, rinsed with MQ water (10 × 1.0 mL).
2. Treat PVDF membranes with 100 µL of Zwittergent 3-16, 0.5% in Tris (0.1 *M*, pH 8.0) and incubate on a shaker at RT for 5 min to block the unoccupied sites on the membrane.
3. Wash membranes extensively with MQ water (10 × 1.0 mL).
4. After blocking the membranes, add appropriate digestion buffer (20 µL) and enzyme of choice (0.2 µg) and start digestion at 37 °C for 5–60 min. **Table 1** lists the digestion conditions of different enzymes.
5. After incubation, add 3 µL of 4% TFA/water to quench the digestion.
6. Rinse membranes with MQ water (10 × 1.0 mL) and dry in the Speed Vac.

Table 1
Digestion conditions of various enzymes (*)

Enzymes	Buffers	Time (min)	Specificity
Trypsin	Tris (0.1 M, pH 8.0)	10	C-terminal side of K & R
Lys-C	Ammonium bicarbonate (50 mM, pH 8.0)	10–30	C-terminal side of K
Clostripain	Tris (0.1 M, pH 8.0) containing 2 mM DTT, 1 mM $CaCl_2$.	10–20	C-terminal side of R
Glu-C	Ammonium bicarbonate (0.1 M, pH 8.)	10–30	C-terminal side of E
Glu-C	Sodium phosphate (50 mM, pH 8.0)	10–30	C-terminal side of E & D
Lys-N	Sodium phosphate (50 mM, pH 8.0)	10–20	N-terminal side of K
Asp-N	Sodium phosphate (50 mM, pH 8.0)	10–20	N-terminal side of D
PGAP	Sodium phosphate (50 mM, pH 7.0) containing 10 mM DTT, 1mM EDTA	60	Removal of pyroglutamyl group

(*) Pyroglutamyl aminopeptidase (PGAP) digestion was performed at 90 °C and the rest was performed at 37 °C

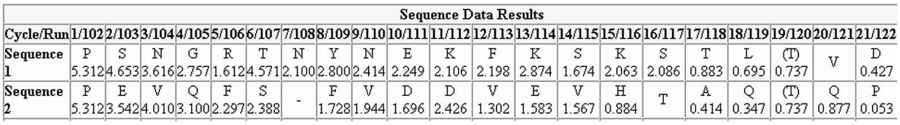
7. Analyze the resultant mixture of peptides as mentioned in **Subheading 3.4** (*see* **Note 5**).

3.6 On-Membrane Digestion for Peptide Mass Fingerprinting (PMF)

1. Block protein bands with Zwittergent solution as described in **Subheading 3.5**.
2. Carefully remove Zwittergent solution and rinse the membrane thoroughly with MQ water (10 × 1.0 mL) (*see* **Note 6**).
3. Heat protein bands to 95 °C for 5-10 min in the appropriate digestion buffer in the presence of 10% acetonitrile (*see* **Notes 7** and **8**).
4. Add the enzyme of choice to the digestion buffer and incubate at 37 °C for 1-3 h.
5. After incubation, quench the reaction with 3 μL of 4% TFA/water.
6. Spot the resultant peptide mixture (0.2-1.0 μL) onto the MALDI target plate using a nitrocellulose-base method (*see* **Note 9**) and air dry.
7. After the organic matrix is dried, wash sample spots with 1 μL of 0.1% TFA/water prior to MALDI analysis to remove any trace amount of salt.
8. Perform PMF on a MALDI-TOF instrument in reflectron mode. (Note, typically we employ the following conditions: mass range is set to 600-6000 Da at a laser power of 1800-1900 AU, acceleration voltage is set to 25 kV.)
9. Mass spectral analysis may be performed using any search algorithm that can perform PMF, for example Mascot (Matrix Sciences, London, UK) or by generating an *in silico* proteolytic digest peak list and manually comparing.

4. Notes

1. If proteins being electroblotted from the gel onto PVDF membrane have a high molecular weight, (> 80 kDa), use CAPS buffer containing 10% of methanol to improve transfer efficiency.
2. When assembling the sandwich cassette, remove all air bubbles from the gel, paper and PVDF, to ensure proper transfer of proteins onto the membrane.
3. Digestion of proteins using dilute acid usually cleaves the bonds between Asp and Pro residues resulting in peptides with a Proline N-terminal residue. However, cleavage between Asp and Gly residues may also be observed. In order to simplify the mixture of generated peptides, especially for proteins with a molecular weight over 80 kDa, one can block peptides starting with a primary amino acid with 30 μL of sodium borate 0.1 M containing 30 mM o-phthaladehyde (OPA) and 57 mM β-mercaptoethanol at RT for 5 min *(5)*.
4. **Fig.1** represents an on-PVDF chemical digestion of anti-OX40 ligand heavy chain using dilute acid solution. Under mild acidic condition, proteins are cleaved at bonds between Asp/Pro residues as shown by proline residue in the first cycle. Treatment of the membrane prior to chemical



Sequence Data Results																					
Cycle/Run	**1/102**	**2/103**	**3/104**	**4/105**	**5/106**	**6/107**	**7/108**	**8/109**	**9/110**	**10/111**	**11/112**	**12/113**	**13/114**	**14/115**	**15/116**	**16/117**	**17/118**	**18/119**	**19/120**	**20/121**	**21/122**
Sequence 1	P 5.312	S 4.653	N 3.616	G 2.757	R 1.612	T 4.571	N 2.100	Y 2.800	N 2.414	E 2.249	K 2.106	F 2.198	K 2.874	S 1.674	K 2.063	S 2.086	T 0.883	L 0.695	(T) 0.737	V	D 0.427
Sequence 2	P 5.312	E 3.542	V 4.010	Q 3.100	F 2.297	S 2.388	-	F 1.728	V 1.944	D 1.696	D 2.426	V 1.302	E 1.583	V 1.567	H 0.884	T	A 0.414	Q 0.347	(T) 0.737	Q 0.877	P 0.053

Fig.1. N-terminal sequence analysis of an on-PVDF Asp/Pro cleavage of anti-OX40 ligand heavy chain at 90 °C for 1h.

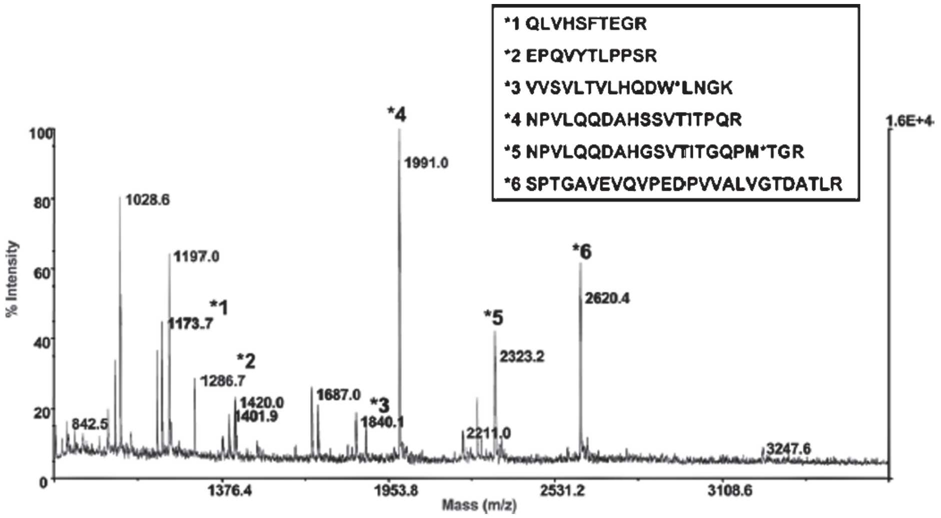
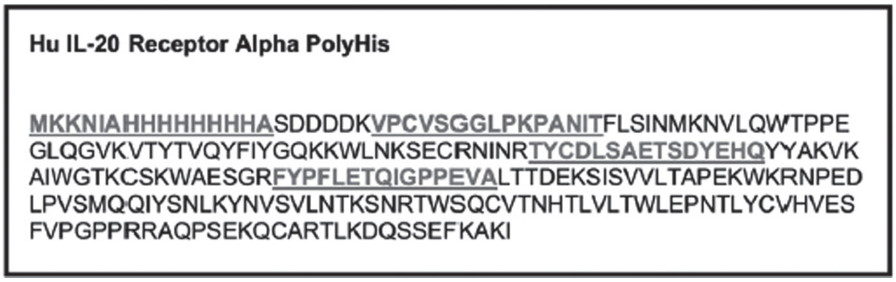


Fig. 2. On-PVDF clostripain digestion of human IL-20 receptor at 37 °C for 20 min. Underlined are peptides generated, which were identified by Edman degradation analysis.

cleavage is not needed. Sequencing data matches the variable and constant region of the heavy chain.

5. Human IL-12 receptor has part of an STII tag, a poly His tag and an enterokinase cleavage site at the N-terminus. Totally the tag consists of 21 amino acids. In order to confirm the identity of this protein by Edman sequencing, one needs to sequence out the first 30 residues in order to get pass the tag, i.e. it would take 10 h (20 min × 30 cycles). Using rapid clostripain digestion, one can confirm the presence of the tag as well as the identity of the protein in much less time **(Fig. 2)**.
6. For peptide mass fingerprinting, it is necessary to remove Zwittergent solution as completely as possible in order to prevent signal suppression in MALDI-TOF analysis. This can be performed by taking a glass Pasteur pipette and repeatedly rinsing the membrane in MQ water.
7. Some researchers have shown that proteolysis of proteins in the presence of mixed organic-aqueous solvents, such as methanol or acetonitrile in



Hu IL-20 Receptor Alpha PolyHis

MKKNIAHHHHHHHHASDDDDKVPCVSGGLPKPANITFLSINMKNVLQWTPPE
GLQGVKVTYTVQYFIYGQKKWLNKSECRNINRTYCDLSAETSDYEHQYYAKVK
AIWGTKCSKWAESGRFYPFLETQIGPPEVALTTDEKSISVVLTAPEKWKRNPED
LPVSMQQIYSNLKYNVSVLNTKSNRTWSQCVTNHTLVLTWLEPNTLYCVHVES
FVPGPPRRAQPSEKQCARTLKDQSSEFKAKI

Fig. 3. PMF mass spectrum of B7H3 receptor after on-PVDF tryptic digestion at 37 °C for 1 h in Tris buffer (0.1 M, pH 8.0) containing 10% acetonitrile.

digestion buffer, helps denature the proteins, which allows more access of the enzyme to the substrate proteins. As a consequence digestion is more effective ***(6)***.

8. Acetonitrile is included in the digestion buffer to help extract generated peptides from the hydrophobic membrane into the solution which can be subsequently analyzed by mass spectrometry analysis without the further clean-up that is necessary if other detergents are used for the extraction step. (**Fig. 3**)
9. If the digestion buffer contains a high concentration of salt (i.e. Asp-N digestion), it is necessary to desalt using a C_{18} ziptip prior to loading the sample onto the MALDI plate for MALDI-TOF analysis. (High salt concentration leads to ion suppression during ionization).

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Enzymatic Digestion of Membrane-Bound Proteins for Peptide Mapping and Internal Sequence Analysis

Joseph Fernandez and Sheenah M. Mische

1. Introduction

Enzymatic digestion of membrane-bound proteins is a sensitive procedure for obtaining internal sequence data of proteins that either have a blocked amino terminus or require two or more stretches of sequence data for DNA cloning or confirmation of protein identification. Since the final step of protein purification is usually SDS-PAGE, electroblotting to either PVDF or nitrocellulose is the simplest and most common procedure for recovering protein free of contaminants (SDS, acrylamide, and so forth) with a high yield. The first report for enzymatic digestion of a nitrocellulose-bound protein for internal sequence analysis was by Aebersold et al. in 1987, with a more detailed procedure later reported by Tempst et al. in 1990 ***(1, 2)***. Basically, these procedures first treated the nitrocellulose-bound protein with PVP-40 (polyvinyl pyrrolidone, M_r 40,000) to prevent enzyme adsorption to any remaining nonspecific protein binding sites on the membrane, washed extensively to remove excess PVP-40, and the sample was enzymatically digested at 37°C overnight. Attempts with PVDF-bound protein using the above procedures ***(3, 4)*** give poor results and generally require >25 µg of protein. PVDF is preferred over nitrocellulose because it can be used for a variety of other structural analysis procedures, such as amino-terminal sequence analysis and amino acid analysis. In addition, peptide recovery from PVDF-bound protein is higher, particularly from higher retention PVDF (ProBlott, Westran, Immobilon P^{sq}). Finally, PVDF-bound protein can be stored dry as opposed to nitrocellulose, which must remain wet during storage and work up to prevent losses during digestion.

Enzymatic digestion of both PVDF- and nitrocellulose-bound protein in the presence of 1% hydrogenated Triton X-100 (RTX-100) buffers as listed in

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Table 1 was first performed after treating the protein band with PVP-40 ***(5)***. Unfortunately, the RTX-100 buffer also removes PVP-40 from the membrane, which can interfere with subsequent reverse-phase HPLC. Further studies ***(6, 7)*** demonstrate that treatment with PVP-40 is unnecessary when RTX-100 is used in the digestion buffer. It appears that RTX-100 acts as both a blocking reagent and a strong elution reagent.

PVDF-bound proteins are visualized by staining and subsequently excised from the blot. Protein bands are immersed in hydrogenated Triton X-100 (RTX-100), which acts as both a reagent for peptide extraction and a blocking reagent for preventing enzyme adsorption to the membrane during digestion. Remaining cystine bonds are reduced with dithiotreitol (DTT) and carboxyamidomethylated with iodoacetamide. After incubation with the enzyme of choice, the peptides are recovered in the digestion buffer. Further washes of the membrane remove the remaining peptides, which can be analyzed by microbore HPLC. Purified peptides can then be subjected to automated sequence analysis.

Additional studies have been reported that have enhanced this procedure. Best et al. ***(8)*** have reported that a second aliquot of enzyme several hours later improves the yield of peptides. Reduction and alkylation of cysteine is possible directly in the digestion buffer, allowing identification of cysteine during sequence analysis ***(9)***. Finally, octyl- or decylglucopyranoside can be substituted for RTX-100 in order to obtain cleaner mass spectrometric analysis of the digestion mixture ***(10)***.

2. Materials

The key to success with this procedure is cleanliness. Use of clean buffers, tubes, and staining/destaining solutions, as well as using only hydrogenated Triton X-100 as opposed to the nonhydrogenated form, greatly reduces contaminant peaks obscured during reverse-phase HPLC. A corresponding blank piece of membrane must always be analyzed at the same time as a sample is digested, as a negative control. Contaminants can occur from may sources and particularly protein contaminants are a cocnern. Human Kertain may contaminate samples if gloves are not worn. Proteins from Western blotting can contaminate the PVDF membrane if previously used dishes are used for staining. UV absorbing contaminants can arise from dirty tubes and sub quality detergents. All solutions should be prepared with either HPLC-grade water or double-glass-distilled water that has been filtered through an activated charcoal filter, and passed through a 0.22-µm filter ***(11)***.

2.1. Preparation of the Membrane-Bound Sample

Protein should be analyzed by SDS-PAGE or 2D-IEF using standard laboratory techniques. Electrophoretic transfer of proteins to the membrane should

Table 1
Digestion Buffers Recipes for Various Enzymes

Enzyme	Digestion buffer	Recipe (using RTX- 100)[a]	Recipe (using OGP)[c]	Comments
Trypsin or Lys-C	1% Detergent/	100 µL 10% RTX-100 stock,	10 mg OGP,	Detergent prevents enzyme adsorption to membrane and increases recovery of peptides
	10% acetonitrile/	100 µL acetonitrile,	100 µL acetonitrile,	
	100 m*M* Tris, pH 8.0	300 µL HPLC-grade water, and	400 µL HPLC-grade water, and	
		500 µL 200 m*M* Tris stock[b]	500 µL 200 m*M* Tris stock[b]	
Glu-C	1% Detergent/	100 µL 10% RTX-100 stock,	10 mg OGP,	Acetonitrile decreases digestion efficiency of Glu-C
	100 m*M* Tris, pH 8.0	400 µL HPLC-grade water, and	500 µL HPLC-grade water, and	
		500 µL 200 m*M* Tris stock[b]	500 µL 200 m*M* Tris stock[b]	
Clostripain	1% Detergent/	100 µL 10% RTX-100 stock,	10 mg OGP,	DTT and $CaCl_2$ are necessary for Clostripain activity
	10% acetonitrile/	100 µL acetonitrile,	100 µL acetonitrile,	
	2 m*M* DTT/	45 µL 45 m*M* DTT,	45 µL 45 m*M* DTT,	
	1 m*M* $CaCl_2$/	10 µL 100 m*M* $CaCl_2$,	10 µL 100 m*M* $CaCl_2$,	

(continued)

Table 1 (continued)

Enzyme	Digestion buffer	Recipe (using RTX- 100)[a]	Recipe (using OGP)[c]	Comments
	100 m*M* Tris, pH 8.0	245 µL HPLC-grade water, and	345 µL HPLC-grade water, and	
		500 µL 200 m*M* Tris stock[b]	500 µL 200 m*M* Tris stock[b]	

[a] Hydrogenated Triton X-100 (RTX-100) as described in Tiller et al. ***(13)***.

[b] 200 m*M* Tris stock (pH 8.0) is made up as follows: 157.6 mg Tris-HCl and 121.1 mg trizma base to a final volume of 10 mL with HPLC-grade water.

[c] Octyl glucopyranoside can be substituted for RTX-100.

be performed in a full immersion tank rather than a semidry transfer system to avoid sample loss and obtain efficient transfer ***(12)***. PVDF membranes with higher protein binding capacity such as Immobilon P^{sq} (Millipore, Bedford, MA), Problott (Applied Biosystems, Foster City, CA), and Westran (Bio-Rad, Hercules, CA), are preferred owing to greater protein recovery on the blot, although all types of PVDF and nitrocellulose can be used with this procedure. The following stains are compatible with the technique: Ponceau S, Amido black, india ink, and chromatographically pure Coomassie brilliant blue with a dye content >90%. **A blank region of the membrane should be excised to serve as a negative control**.

2.2. Enzymatic Digestion Buffers

Digestion buffer should be made as described in **Table 1**. Make up 1 mL of buffer at a time and store at –20°C for up to 1 wk. Hydrogenated Triton X-100 (RTX-100) (protein grade, Calbiochem, LaJolla, CA) is purchased as a 10% solution, which should be stored at –20°C. Note: Only hydrogenated Triton X-100 should be used since UV-absorbing contaminants are present in ordinary Triton X-100, making identification of peptides on subsequent HPLC impossible (*see* **Fig. 1**). Alternately, octyl glucopyranoside (OGP) (Ultrol-grade, Calbiochem) or decyl glucopyranoside (DGP) (Ultrol-grade, Calbiochem) can be substituted for RTX-100 as described in **Table 1**.

2.3. Reduction and Carboxyamidomethylation

1. 45 m*M* DTT: bring 3.5 mg DTT (Ultrol- grade, Calbiochem) up in 500 µL HPLC-grade water. This can be stored at –20°C for up to 3 mo.
2. 100 m*M* iodoacetamide solution: bring 9.25 mg iodoacetamide (reagent-grade) up in 500 µL HPLC-grade water. This solution must be made fresh just prior to use. Dry DTT and iodoacetamide should be stored at 4°C or –20°C.

2.4. Enzyme Solutions and Inhibitors

Enzymes should be stored as small aliquots at –20°C, and made up as 0.1 µg/µL solutions immediately before use. These aliquots can be stored for at least 1 mo at –20°C without significant loss of enzymatic activity.

1. Trypsin (25 µg, sequencing-grade, Boehringer Mannheim, Indianapolis, IN): Solubilize trypsin in 25 µL of 0.01% trifluoroacetic acid (TFA), and let stand ~10 min. Aliquot 5 µL (5 µg) quantities to clean microcentrifuge tubes, dry in a SpeedVac, and store at –20°C. Reconstitute the dry enzyme in 50 µL 0.01% TFA for a 0.1 µg/µL working solution, which is good for 1 d. **Trypsin cleaves at arginine and lysine residues**.
2. Endoproteinase Lys-C (3.57 mg, Wako Pure Chemicals, Osaka, Japan): Solubilize enzyme in 1000 µL HPLC- grade water, and let stand ~10 min. Make nine 100-µL

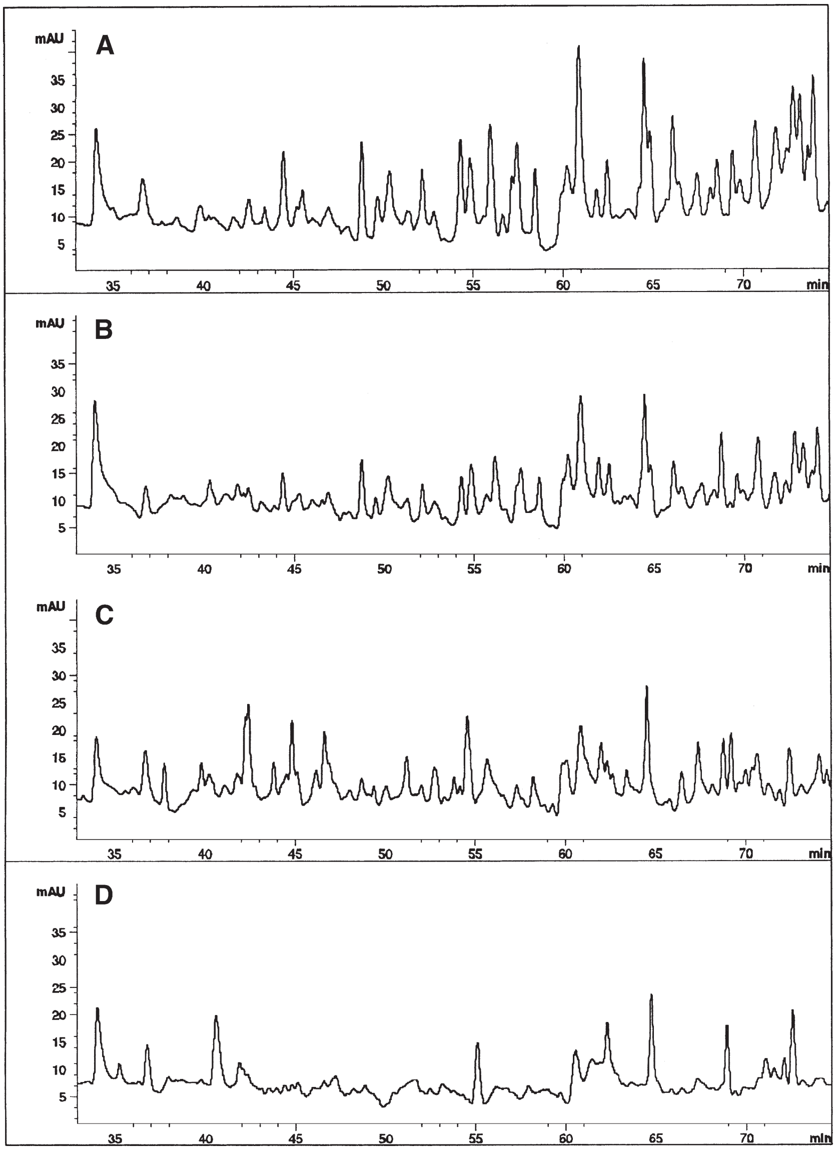

Fig. 1. Peptide maps of trypsin digestion of beta galactosidase bound to PVDF. Panels A–D represent varying amounts of proteins, 40 pmol, 20 pmol, 10 pmol, and 5 pmol respectively. Proteins were analyzed by SDS-PAGE, transferred to PVDF, and stained with Coomassie Brilliant Blue G-250.

quantities to clean tubes, and store at –20°C. Disperse remaining 100 µL into 20 × 5 µL aliquots (17.85 µg each), and store at –20°C. When needed, take one 5-µL aliquot and add 173 µL of HPLC-grade water to establish a 0.1 µg/µL working solution, which can be used for up to 1 wk if stored at –20°C between uses. When 5-µL aliquots are used up, disperse another 100-µL aliquot. **Endoptoteinase Lys-C cleaves only at lysine residues**.

3. Endoproteinase Glu-C (50 µg, sequencing- grade, Boehringer Mannheim) : Solubilize in 100 µL of HPLC- grade water, and let stand ~10 min. Aliquot 10-µL (5 µg) quantities to clean tubes, dry in a SpeedVac, and store at –20°C. Reconstitute the enzyme in 50 µL HPLC-grade water for a 0.1 µg/µL solution, which is good for only 1 d. **Under these conditions, endoproteinase Glu-C cleaves predominantly at glutamic acid residues, but can sometimes cleave at aspartic acid residues**.
4. Clostripain (20 µg, sequencing-grade, Promega, Madison, WI): Solubilize enzyme in 200 µL of manufacturer's supplied buffer for a concentration of 0.1 µg/µL. Enzyme solution can be stored at –20°C for 1 mo. **Clostripain cleaves at arginine residues only**.
5. 1% Diisopropyl fluorophosphate (DFP) solution: DFP is a dangerous neurotoxin that must be handled with double gloves in a chemical hood. Please follow all precautions listed with this chemical. Add 10 µL of DFP to 990 µL absolute ethanol in a capped microcentrifuge tube. Store at –20°C.

3. Method

3.1. Preparation of the Membrane-Bound Sample

Protein should be electrophoresed in one or two dimensions, followed by electrophoretic transfer to the membrane and visualization of the bands according to the following suggestions.

1. Electroblotting of proteins will be most efficient with a tank transfer system, rather than a semidry system. Concentrate as much protein into a lane as possible; however, if protein resolution is a concern, up to 5 cm^2 of membrane can be combined for digestion (*see* **Notes 3** and **5**).
2. Stain PVDF membrane with either Ponceau S, Amido black, india ink, or chromatographically pure Coomassie brilliant blue with a dye content of 90%. Destain the blot until the background is clean enough to visualize the stained protein. Complete destaining of the blot is unnecessary, but at least three washes (~5 min) with distilled water should be done to remove excess acetic acid, which is used during destaining (*see* **Note 5**).
3. Excise protein band(s), and place into a clean 1.5-mL microcentrifuge tube. In addition, excise a blank region of the membrane approximately the same size as the protein blot to serve as a negative control (*see* **Note 4**).
4. Air dry PVDF-bound protein dry at room temperature and store at –20°C or 4°C.

3.2. Digestion of the Membrane-Bound Protein

NOTE: Gloves should be worn during all steps to avoid contamination of sample with skin keratin.

1. Place ~100 µL of HPLC-grade water onto a clean glass plate, and submerge the membrane-bound protein into the water. Transfer the wet membrane to a dry region of the plate and with a clean razor blade, cut the membrane first lengthwise into 1-mm wide strips, and then perpendicular so that the membrane pieces are 1 × 1 mm. Treat the negative control under the same conditions as the sample. Keeping the membrane wet will simplify manipulation of the sample as well as minimize static charge, which could cause PVDF to "jump" off the plate. The 1 × 1 mm pieces of membrane will settle to the bottom of the tube and require less digestion buffer to immerse the membrane completely (*see* **Note 7**).
2. Slide the cut membrane onto the forceps with the razor blade, and return it to a clean 1.5-mL microcentrifuge tube. Use the cleanest tubes possible to minimize contamination during peptide mapping. Surprisingly, many UV-absorbing contaminants can be found in microcentrifuge tubes, and this appears to vary with supplier and lot number. Tubes can be cleaned by rinsing with 1 mL of HPLC-grade methanol followed by 2 rinses of 1 mL HPLC-grade water prior to adding the protein band.
3. Add 50 µL of the appropriate digestion buffer (**Table 1**), and vortex thoroughly for 10–20 s. Optionally, add 50 µL digestion buffer to an empty microcentrifuge tube to serve as a digestion blank for HPLC analysis. The amount of digestion buffer can be increased or decreased depending on the amount of membrane; however, the best results will be obtained with a minimum amount of digestion buffer. PVDF membrane will float in the solution at first, but will submerge after a short while, depending on the type and amount of PVDF.
4. Add 5 µL of 45 mM DTT, vortex thoroughly for 10–20 s, seal the tube cap with parafilm, and incubate at 55°C for 30 min. DTT will reduce any remaining cystine bonds. DTT should be of the highest grade to reduce uv absorbing contaminants that might interfere with subsequent peptide mapping.
5. Allow the sample to cool to room temperature. Add 5 µL of 100 mM iodoacetamide, vortex thoroughly for 10–20 s, and incubate at room temperature for 30 min in the dark. Iodoacetamide alkylates cysteine residues to generate carboxyamidomethyl cysteine, allowing identification of cysteine during sequence analysis. Allowing the sample to cool prior to adding iodoacetamide and incubating at room temperature are necessary to avoid side reaction to other amino acids.
6. Add enough of the required enzyme solution to obtain an estimated enzyme to substrate ratio of 1:10 (w/w) and vortex thoroughly for 10–20 s. Incubate the sample (including digestion buffer blank) at 37°C for 22–24 h. The amount of protein (substrate) can be estimated by comparison of staining intensity to that of known quantities of stained standard proteins. The 1:10 ratio is a general guideline. Ratios of 1:2 through 1:50 can be used without loss of enzyme efficiency or peptide recovery. An enzyme should be selected that would likely produce peptides of > 10 amino acids long. Amino acid analysis of the protein would be informative for estimating the number of cleavage sites. A second aliquot of enzyme can be added after 4–6 h (*see* **Note 6**).

3.3. Extraction of the Peptides

1. After digestion, vortex the sample for 5–10 s, sonicate for 5 min by holding in a sonicating water bath, spin in a centrifuge (~1800g) for 2 min, and transfer the supernatant to a separate vial that will be used directly for HPLC analysis.
2. Add a fresh 50 µL of digestion buffer to the sample, repeat **step 11**, and pool the supernatant with the original buffer supernatant.
3. Add 100 µL 0.1% TFA to the sample, and repeat **step 1**. The total volume for injection onto the HPLC is 200 µL. Most of the peptides (~80%) are recovered in the original digestion buffer; however, these additional washes will ensure maximum recovery of peptides from the membrane.
4. Terminate the enzymatic reaction by either analyzing immediately by HPLC or adding 2 µL of the DFP solution.

CAUTION: DFP is a dangerous neurotoxin and must be handled with double gloves under a chemical fume hood. Please follow all precautions listed for this chemical.

3.4. Analysis of Samples by Reverse-Phase HPLC and Storage of Peptide Fractions

1. Prior to reverse-phase HPLC, inspect the pooled supernatants for small pieces of membrane or particles that could clog the HPLC tubing. If membrane or particles are observed, either remove the membrane with a clean probe (such as thin tweezers, a thin wire, thin pipet tip, and so forth), or spin in a centrifuge for 2 min and transfer the sample to a clean vial. A precolumn filter will help increase the life of HPLC columns, which frequently have problems with clogged frits.
2. Sample is ready to be fractionated by HPLC (*see* Chapter 102). Fractions can be collected in capless 1.5-mL plastic tubes, capped, and stored at –20°C until sequenced (*see* **Note 9**). A typical fractionation is shown in **Fig. 1**.

4. Notes

1. This procedure is generally applicable to proteins that need to have their primary structure determined and offers a simple method for obtaining internal sequence data in addition to amino terminal sequence analysis data. The procedure is highly reproducible and is suitable to peptide mapping by reverse-phase HPLC. Proteins 12–300 kDa have been successfully digested with this procedure with the average size around 100 kDa. Types of proteins analyzed by this technique include DNA binding, cystolic, peripheral, and integral-membrane proteins, including glycosylated and phosphorylated species. The limits of the procedure appear to be dependent on the sensitivity of both the HPLC used for peptide isolation and the protein sequencer.
2. There are several clear advantages of this procedure over existing methods. First, it is applicable to PVDF (especially high-retention PVDF

membranes), which is the preferred membrane owing to higher recovery of peptides after digestion, as well as being applicable to other structural analysis. The earlier procedures ***(1,2)*** have not been successful with PVDF. Second, because of the RTX-100 buffer, recovery of peptides from nitrocellulose is higher than earlier nitrocellulose procedures ***(5)***. Third, the procedure is a onestep procedure and does not require pretreatment of the protein band with PVP-40. Fourth, since the procedure does not require all the washes that the PVP-40 procedures do, there is less chance of protein washout. Fifth, the time required is considerably less than with the other procedures. Overall, the protocol described here is the simplest and quickest method to obtain quantitative recovery of peptides.

3. The largest source of sample loss is generally not the digestion itself, but rather electroblotting of the sample. Protein electroblotting should be performed with the following considerations. Use PVDF (preferably a higher binding type, i.e., ProBlott, Immobilon P^{sq}) rather than nitrocellulose, since peptide recovery after digestion is usually higher with PVDF ***(5,8)***. If nitrocellulose must be used, e.g., protein is already bound to nitrocellulse before digestion is required, never allow the membrane to dry out since this will decrease yields. Always electroblot protein using a transfer tank system, since yields from semidry systems are not as high ***(12)***. Using stains such as Ponceau S, Amido black, or chromatographically pure Coomassie brilliant blue, with a dye content >90% will increase detection of peptide fragments during reverse-phase HPLC.

   Note: Most commercial sources of Coomassie brilliant blue are extremely dirty and should be avoided. Only chromatographically pure Coomassie brilliant blue with a dye content of 90% appears suitable for this procedure.

4. The greatest source of failure in obtaining internal sequence data is not enough protein on the blot, which results in the failure to detect peptide during HPLC analysis. An indication of insufficient protein is that either the intensity of the stain is weak, i.e., cannot be seen with Amido black even though observable with india ink (about 10-fold more sensitive), or possibly detectable by radioactivity or immunostaining, but not by protein stain. Amino acid analysis, amino-terminal sequence analysis, or at the very least, comparison with stained standard proteins on the blot should be performed to help determine if enough material is present. When <10 μg of protein is present, the most problematic item is misidentification of peptides on reverse-phase HPLC owing to artifact peaks and contaminants. Although elimination of every contaminant is usually impossible, there are several strategic points and steps that can be taken to help alleviate these

contaminants. A negative control of a blank region of the membrane blot (preferably from a blank lane) that is approximately the same size as the protein band will help to identify contaminants present that are associated with the membrane and digestion buffer (*see* **Fig. 1D**). The blank membrane should have gone through the same preparation steps as the sample, including electroblotting and staining, and should be analyzed by HPLC immediately before or after the sample. A positive control (membrane-bound standard protein) is generally unnecessary, but should be performed if the activity of the enzyme is in question or a new lot number of enzyme is to be used.

5. Major sources of contaminants are stains used to visualize the protein, the microcentrifuge tubes used for digestion, reagents used during digestion and extraction of peptides, and the HPLC itself. Stains are the greatest source of contaminants, and Coomassie brilliant blue in particular is a problem (*see* **Fig. 1A**). Amido black and Ponceau S are generally the cleanest, whereas chromatographically pure Coomassie brilliant blue with a dye content <90% does appear to generate less contaminants than most other commercially available Coomassie brilliant blue stains. Surprisingly, microcentrifuge tubes can produce significant artifact peaks, which seem to vary with supplier and lot number. A digestion blank of just the microcentrifuge tube should be done, since some contaminants only appear after incubation in the RTX-100 buffer. The major concern with the digestion buffer is the hydrogenated Triton X-100 (*see* **Fig. 1C**). Additional late-eluting peaks may be observed with certain lots of RTX-100, whereas other lots are completely free of UV-absorbing contaminants. HPLC-grade water or water prepared as described by Atherton ***(11)*** should be used for all solution preparation. An HPLC blank (gradient run with no injection) should always be performed to determine what peaks are related to the HPLC (*see* **Fig. 1E**).
6. The key to success of the procedure and quantitative recovery of peptides from both PVDF and nitrocellulose membranes is the use of hydrogenated Triton X-100 (RTX-100) in the buffer. This should be purchased from Calbiochem as a 10% stock solution, protein-grade (cat. no. 648464). **Figure 1C** demonstrates why hydrogenated Triton X-100 should be used, since nonhydrogenated Triton X-100 has several strong UV-absorbing contaminants ***(13)***. RTX-100 acts as a block to prevent enzyme adsorption to the membrane as well as a strong elution reagent of peptides ***(6,7)***. In addition, RTX-100 does not inhibit enzyme activity or interfere with peak resolution during HPLC, as do ionic detergents, such as SDS ***(5)***. The concentration of RTX-100 can be decreased to 0.1% ***(7)***. However, with a large amount of membrane, there could be a decrease in peptide recovery. Optionally,

octyl- or decylglucopyranoside can be substituted for RTX-100 with no loss in peptide recovery ***(10)***.

7. The membrane should be cut into 1 × 1 mm pieces while keeping it wet to avoid static charge buildup. The 1 × 1 mm pieces allow using the minimum volume of digestion buffer to cover the membrane. The volume of the digestion buffer should be enough to cover the membrane (~50 µL), but can be increased or decreased depending on the amount of membrane present. The enzyme solution should be selected based on additional knowledge of the protein, such as amino acid composition or whether the protein is basic or acidic. If the protein is a complete unknown, endoproteinase Lys-C or Glu-C would be a good choice. The enzyme-to-substrate ratio should be about 1:10; however, if the exact amount of protein is unknown, ratios of 1:2 through 1:50 will not affect the quantitative recovery of peptides. After digestion, most of the peptides are recovered in the original buffer (about 80%), and the additional washes are performed to ensure maximum peptide recovery. Microbore reverse-phase HPLC is the best isolation procedure for peptides.
8. As mentioned earlier, previous procedures ***(1–3, 5)*** require pretreatment with PVP-40 to prevent enzyme adsorption to the membrane. RTX-100 is essential for quantitative recovery of peptides from the membrane; however, RTX-100 also strips PVP-40 from the membrane, resulting in a broad, large UV-absorbing contaminant that can interfere with peptide identification. The PVP-40 contaminant is not dependent on the age or lot number of PVP-40, and making fresh solutions did not help as previously suggested ***(4)***. This appears to be more of a problem with nitrocellulose and higher binding PVDF (ProBlott and Immobilon P^{sq}) than lower binding PVDF (Immobilon P), and also is dependent on the amount of membrane. The PVP-40 contaminant also appears to elute earlier in the chromatogram as the HPLC column ages, becoming more of a nuisance in visualizing peptides. Therefore, using PVP-40 to prevent enzyme adsorbtion to the membrane should be avoided.
9. There are a few considerations that should be addressed regarding peptide mapping by HPLC using the protocol described here. A precolumn filter (Upchurch Scientific) must be used to prevent small membrane particles from reaching the HPLC column, thus decreasing its life. Inspection of the pooled supernatants for visible pieces of PVDF can prevent clogs in the microbore tubing, and can be removed either with a probe, or by spinning in a centrifuge and transferring the sample to a clean vial.

   Peptide mapping by reverse-phase HPLC after digestion of the membrane-bound protein should result in several peaks on the HPLC. Representative peptide maps from trypsin digestions of human transferrin bound to PVDF and stained with Coomassie blue or amido black (Immobilon P^sq^) is shown in Figs. 1A and B. Peptide maps should be reproducible under identical digestion and HPLC conditions. In addition, the peptide maps from proteins digested on membranes are comparable if not identical to those digested in solution, indicating that the same number of peptides are recovered from the membrane as from in solution. The average peptide recovery is generally 40–70% based on the amount analyzed by SDS-PAGE, and 70–100% based on the amount bound to PVDF as determined by amino acid analysis or radioactivity counting. The recovery of peptides from the membrane appears to be quantitative, and the greatest loss of sample tends to be in the electroblotting.

10. The entire procedure can be done in approx 24 h plus the time required for peptide mapping by reverse-phase HPLC. Cutting the membrane takes about 10 minutes, reduction with DTT takes 30 min, carboxyamidomethylation take another 30 min, digestion at 37°C takes 22–24 h, and extraction of the peptides requires about 20 min.

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Reverse-Phase HPLC Separation of Enzymatic Digests of Proteins

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1. Introduction

Reverse-phase HPLC is a powerful technique having the ability to resolve complex mixtures of peptides within a short amount of time, in volatile solvents and is compatible with mass spectrometric approaches. Recent technological advances in HPLC analysis include improved stationary phase packing materials for columns (moving from 10 µm to 3 µm and now 1.7 µm particle sizes) and next generation LC systems that have improved resolution, speed and sensitivity and operate at ultra high pressures of up to 10,000 psi ***(1)***. These LC systems are commonly referred to as ultra high pressure or ultra performance liquid chromatography (UPLC). In general, we find that peptides that are less than about 30 residues in length usually separate based on their content of hydrophobic amino acids and that their relative elution positions can be reasonably accurately predicted from published retention coefficients ***(2,3)***. Since proteins often retain some degree of folding under the conditions used for reverse-phase HPLC, the more relevant parameter in this instance is probably surface rather than total hydrophobicity. Although larger peptides and proteins may be separated on HPLC, sometimes their tight binding, slow kinetics of release, propensity to aggregate, and relative insolubility in the usual acetonitrile/0.05% trifluoroacetic acid mobile phase results in broad peaks and/or carryover to successive chromatograms. In our experience, these problems are seldom seen with peptides that are less than about 30 residues in length, which thus makes reverse-phase HPLC an ideal method for fractionating enzymatic digests of proteins. Although it is sometimes possible to improve a particular separation by lessening the gradient slope in that region of the chromatogram, generally, enzymatic digests from a wide variety of proteins can be reasonably well fractionated using a single

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gradient that might extend over 1–2 h. Another advantage of reverse-phase HPLC is its excellent reproducibility which greatly facilitates using comparative HPLC peptide mapping to detect subtle alterations between otherwise identical proteins. Applications of this approach might include identifying point mutations as well as sites of chemical and posttranslational modification and demonstrating precursor/ product relationships. Finally, since peptides are isolated from reverse-phase HPLC in aqueous mixtures of acetonitrile and 0.05% to 0.1% of an acid (typically TFA, acetic acid, or formic acid) they are ideally suited for subsequent analysis by matrix-assisted laser desorption mass spectrometry (MALDI-MS, ***10***), on-line LC-MS/MS and automated Edman sequencing.

2. Materials

1. HPLC system: Digests of 1 pmol to 10 nmol amounts of proteins may be fractionated on a Hewlett Packard 1090M, 1100 or comparable analytical HPLC system (*see* **Note 1** for suggestions on evaluating HPLC systems and for a general discussion of important parameters that affect HPLC reproducibility, resolution, and sensitivity) capable of generating reproducible gradients in the flowrate range extending at least from about 50 to 500 µL/min. The HP 1090M HPLC used routinely in our lab for off line LC analysis, was equipped with an optical bench upgrade (HP #79891A), a 1.7-µL high-pressure microflow cell with a 0.6-cm path length, a diode array detector, a Waters Chromatography static mixer, and a 250-µL injection loop. The detector outlet was connected to an Isco Foxy fraction collector and Isco Model 2150 Peak Separator to permit collection by peaks into 1.5-mL Sarstedt capless Eppendorf tubes that were positioned in the tops of 13 × 100 mm test tubes. We have found that by applying a varying resistance in parallel to the input detector signal that enters the Model 2150 Peak Separator, it is possible to improve peak detection significantly in the <500 pmol range. This can be easily accomplished via a small "black box" that is equipped with a four-position switch and that contains four different resistors labeled and configured as follows: 50 pmol/2,000 Ω, 100 pmol/1000 Ω, 250 pmol/470 Ω, 500 pmol/220 Ω. With this arrangement, best peak detection is obtained with the following settings on the 2150 Peak Separator: input = 10 mV, peak duration = 1 min, slope sensitivity = high. To minimize the "transit time" during which a given peak is traveling from the flow cell to the fraction collector, these two components are connected with 91 cm of 75-µ id fused silica capillary tubing (*see* **Note 2**). With this configuration, the dead volume is equal to about 7.5 µL, which corresponds to a peak delay of about 9 s at a flowrate of 50 µL/min, and the drop size is sufficiently small that even extremely small peak volumes can be accurately fractionated.
2. A 5-µ particle size, 1 × 250 mm C18 microbore column is recommended for fractionating (off line) 1–250 pmol amounts of digests, whereas 250 pmol to 10 nmol amounts may be fractionated on 3.9–4.6 mm id analytical columns. Although many commercially available columns would undoubtedly be satisfactory (*see* **Note 3**), the column that is currently being used in our laboratory for off line HPLC analysis is the 300-Å Vydac C18 (cat. # 218TP51 for the 1-mm column, Separations

Group, Hesperia, CA) and the RP-300 Aquapore C8, 7 µm, 300-Å, 1 × 250 mm id Brownlee Microbore Column, (cat. # 07120097, PerkinElmer)

3. For on-line LC analysis, a capillary LC system such as the Waters cap LC, Dionex Ultimate or the Waters nanoAcquity UPLC is recommended. Normal HPLC systems as described above, can also be used if stream splitting is enabled so that the flow rate into the mass spectrometer is appropriate for the column and electrospray source used.
4. For the Waters capLC and the Dionex Ultimate systems, we use a Waters Atlantis™ dC18, 3 µm particle size, 100-Å pore size, 100 µm (or 75 µm) × 150 mm NanoEase™ column. The flow rate into the mass spectrometer is 300 nl/min for the 75 µm column and 500 nl/min for the 100 µm column. On the Waters nanoAcquity UPLC system, a 1.7 µm, 75 µm × 250 mm nanoAcquity UPLC column is used. Typical flow rate is 300 nl/min. (*see* **Note 4)**
5. pH 2.0 Buffer system off line analysis: *see* **Notes 5** and **6**. Buffer A: 0.06% trifluoroacetic acid (TFA) (3 mL 20% TFA/H_2O/L final volume HPLC-grade H_2O). Buffer B: 0.052% TFA/80% acetonitrile (2.7 mL 20% TFA/H_2O, 800 mL CH_3CN [HPLCgrade], HPLC-grade H_2O to a final volume of 1000 mL).
6. pH 6.0 Buffer system, off line analysis: *see* **Note 6**. Buffer A: 5 m*M* potassium phosphate, pH 6.0 (10 mL 0.5*M* KH_2PO_4 in a total volume of 1000 mL HPLC-grade H_2O). Buffer B: 80% (v/v) CH_3CN (200 mL HPLC-grade H_2O and 800 mL acetonitrile [HPLC-grade]).
7. CH_3COOH Buffer system for LC-MS analysis: Buffer A: 98% H_2O, 2%CH_3CN, 0.1% CH_3COOH (100 µl % CH_3COOH [HPLC grade], plus 2mL CH_3CN [HPLCgrade] diluted to 100 mL with HPLC grade H_2O) Buffer B: 20% H_2O, 80% CH_3CN, 0.09% CH_3COOH (100 µl % CH_3COOH [HPLC grade], plus 20mL H_2O [HPLC grade] diluted to 100mL with CH_3CN [HPLCgrade])
8. HCOOH Buffer system for Waters nanoAcquity UPLC: Buffer A: 100% H_2O, 0.1% HCOOH (100 µl 99+% HCOOH [Pierce], diluted to 100 mL with HPLC grade H_2O) Buffer B: 100% CH_3CN, 0.075% (75 µl HCOOH (100 µl 99+% HCOOH [Pierce], diluted to 100mL with CH_3CN [HPLCgrade])
9. Peptide dilution buffer: 2 *M* urea, 0.1 *M* NH_4HCO_3. Dissolve 1.2 g Pierce Sequanal Grade urea and 79 mg NH_4HCO_3 (Mallinckrodt Baker) in a final volume of 10 mL HPLC-grade H_2O. This solution should be made up at least weekly and stored at −20 °C.

3. Methods

3.1. HPLC Separation of Peptides

The TFA acetonitrile buffer system described in **Materials**, **item 5** is an almost universal reverse-phase solvent system owing to its low-UV absorbance, high resolution (minimizing peak broadening), and excellent peptide solubilizing properties. However, for HPLC analysis on line with an electrospray mass spectrometer, the TFA needs to be greatly minimized or removed. This is because the TFA has a suppressive effect on the analyte signal due to a combined effect of ion-pairing and surface tension modifications. ***(4)*** Newer generations of columns (e.g. Waters Atlantis dC18 columns) no longer require TFA to maintain peak shape or retention, and formic acid or acetic acid can be used instead making these columns MS compatible. The gradients we generally use are:

Normal Gradient for HPLC Separations		Fast Gradient used on UPLC	
Time	% B	Time	%B
0–60 min	2–37.5%	0–1 min	1%
60–90 min	37.5–75%	1–50 min	1–50%
90–105 min	75–98%	50–51 min	50–85%

In the case of extremely complex digests (i.e., tryptic digests of proteins that are above about 100 kDa), the gradient times may be doubled. In general, we recommend using the lowest flowrates consistent with near-optimum resolution for the column diameter that is being used ***(5,6)***. Hence, we recommend a flowrate of 250–300 nl/min for 75 µm and 500nl/min for 100 µm capillary columns; 50 µL/min for 1mm Microbore columns; 75 µL/min for 2.0–2.1 mm Narrowbore columns; and a flowrate of 0.5 mL/min for 3.9–4.6 mm Analytical columns.

For digests analyzed off line from a mass spectrometer, the peptides can be collected for further analysis such as MALDI-MS or edman sequencing. For these samples, all fractions are immediately tightly capped (to prevent evaporation of the acetonitrile—*see* **Note 7**) and are then stored in plastic boxes (USA/Scientific Plastics, Part #2350–5000) at 5 °C. With the reduced flowrate of 50 µL/min, the average peak detected fraction volume is about 35 µL, which is sufficiently small that, if necessary, the entire fraction can be directly spotted onto support disks used for automated Edman degradation. For MALDI-MS analysis, 1 µL of the fraction can be removed and analyzed directly. To prevent adsorptive peptide losses onto the plastic tubes, fractions should not be concentrated prior to further analysis and, after spotting the peptide sample for edman sequencing, the empty tube should be rinsed with 50 µL 100% TFA, which is then overlaid on top of the sample. If <100% of the sample is to be sequenced, we recommend that the fraction that is to be saved be transferred to a second tube, so that the tube in which the sample was collected can be rinsed in the same manner as described with 100% TFA. Because one of the important applications of reverse-phase HPLC is comparative peptide mapping, we have included below (*see* **Note 8**) a brief discussion of the use of this approach to identifying subtle structural modifications between otherwise identical proteins.

3.2. HPLC Repurification of Peptides

Peptides from novel proteins whose absorbance profile and/or MALDI-MS spectrum indicate they are insufficiently pure for edman chemical sequencing, may be further purified by chromatography on a second (different) C18 column developed with the same mobile phase and gradient as was used for the initial separation. Because of their differing selectivity ***(7)***, we recommend that (when necessary) peptides that are initially separated on a Vydac C-18 column be further purified by injection onto an Aquapore C8 that is eluted with the same mobile phase and gradient as was used for the initial separation. Peptides destined for repurification are mixed with a volume of $2M$ urea, $0.1M$ NH_4HCO_3 such that the volume of the $2M$ urea, $0.1M$ NH_4HCO_3 is equal to or greater than the volume of 0.05% TFA, acetonitrile in which the fraction was originally isolated (*see* **Note 9**). In this way, the acetonitrile concentration is diluted by at least 50%, which, in our experience, is sufficient to permit peptide binding to the second C-18 column.

In those few instances where the sequential use of Vydac C-18 and Aquapore C-8 columns fails to bring about sufficient purification, the sample may be further purified by chromatography at pH 6.0 on either of these columns (*see* **Note 10**). Again, the same gradient is used with the only difference being the change in mobile phase.

4. Notes

1. Although general suggestions for selecting suitable HPLC systems may be found in Stone et al. ***(6)***, three factors that critically impact on peptide HPLC and that will be briefly discussed are reproducibility, resolution, and sensitivity. Although reproducibility will not have a significant impact on the success of a single analytical HPLC separation, comparative peptide mapping requires that successive chromatograms of digests of the same protein be sufficiently similar that they can be overlaid onto one another with little or no detectable differences. In general, the latter requires that average peak retention times not vary by more than about ± 0.20% ***(6)***. Assuming the digests were carried out under identical conditions, problems with regard to reproducibility often relate to the inability of the HPLC pumps to deliver accurate flowrates at the extremes of the gradient range. That is, to accurately deliver a 99% buffer A/1% buffer B composition at an overall flowrate of 75 µL/min requires that pump B be able to accurately pump at a flowrate of only 0.75 µL/min. The latter is well beyond the capabilities of many conventional HPLC systems. Although reproducibility can be improved somewhat by restricting the gradient range to 2–98%, as opposed to 0–100% buffer B, the reproducibility of each HPLC system will be inherently limited in this regard by the ability of its pumps to deliver low flowrates accurately. Obviously, some HPLC systems that provide reproducible chromatograms at an overall flowrate of 0.5 mL/min might be unable to do so at 75 µL/min ***(6)***. Similarly, minor check valve, piston seal, and injection valve leaks that go unnoticed at 0.5 mL/min might well account for reproducibility problems at 75 µL/min. With the newer generation of ultra performance LC systems (e.g Waters Acquity and nanoAcquity UPLC) reproducibility is not an issue

with run to run variation at less than ±0.2% even when requested to run at 300 nl/min at 99% buffer A.

The ability of HPLC to discriminate between chemically similar peptides and to resolve adequately a reasonable number of peptides from a high-mol-wt protein, which might well produce 100 or more tryptic peptides, is critically dependent on resolution, which, in turn, depends on a large number of parameters, including the flowrate, gradient time, column packing, and dimensions as well as the mobile phase ***(5–7)***. Studies with tryptic digests of transferrin suggest that, within reasonable limits, gradient time is a more important determinant of resolution than is gradient volume. In general, a total gradient time of ~100 min seems to represent a reasonable compromise between optimizing resolution and maintaining reasonable gradient times ***(5)***. And, unless precautions are taken to minimize dead volumes, significant problems may be encountered in terms of automated peak detection/collection and postcolumn mixing as flowrates are lowered much below 0.15 mL/min ***(7)***. Typically, the use of flowrates in the 25–75 µL/min range require that fused silica tubing be used between the detector and the fraction collector, and that a low volume flow cell (i.e., 1–2 µL) be substituted for the standard flow cell in the UV detector.

Since peptide resolution appears to be directly related to column length ***(5–7)***, whenever possible, the 250-mm versions of these columns should be used. The caveat is that as the inner diameter of the column is reduced to 75 or 100 µm, the column length typically has to be shortened in order to keep the pressure at levels low enough to prevent a high pressure shut down. The new ultraperformance LC systems operate at much higher pressures and now, 75 µm × 250 mm columns can be run at 300 nl/min with pressures of approximately 6000 psi (*see* **Fig 1**). Although the low-UV absorbance, high resolution, and excellent solubilizing properties of the 0.05% TFA/acetonitrile, pH 2.0, buffer system have made it the almost universal mobile phase for reverse-phase HPLC, there are occasions when a different mobile phase might be advantageous. Hence, the differing selectivity of the 5 m*M,* pH 6.0, phosphate system ***(7)*** makes this a valuable mobile phase for detecting posttranslational modifications (such as deamidation) that may be more difficult to detect at the lower pH of the TFA system (where ionization of side-chain carboxyl groups would be suppressed). In addition, as noted in **Subheading 3.2**, changing the mobile phase provides another approach for further purifying peptides that were originally isolated in the TFA system. In our experience, however, the pH 2.0 mobile phase provides somewhat better resolution than the higher pH mobile phase ***(7)***. Hence, we recommend using the pH 2.0 system for the initial separation.

The sensitivity of detection of HPLC is dictated primarily by the volume in which each peak is eluted. Although sensitivity can be increased

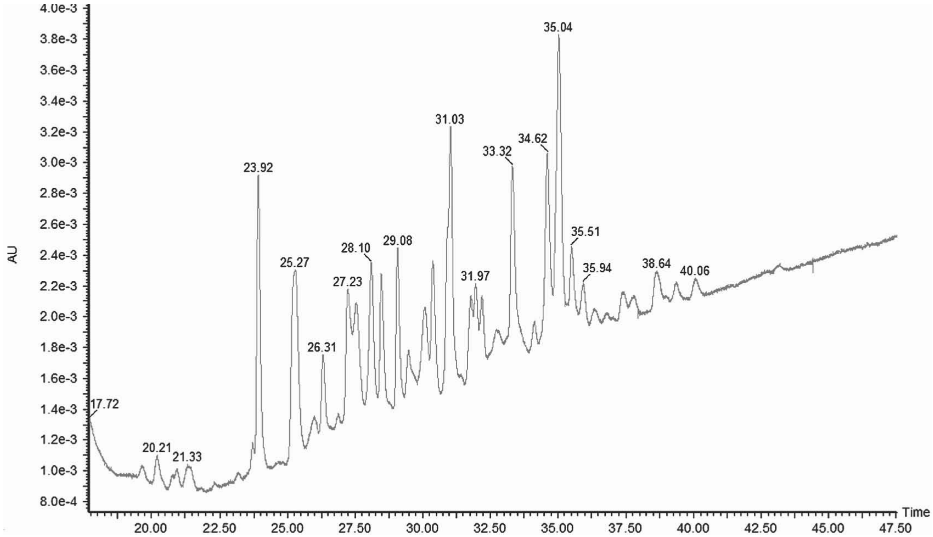

Fig. 1. Reverse-phase HPLC of a 250 fmol aliquot of a large scale, in solution digest of carbamidomethylated transferrin. 250 fmol of the transferrin digest was analyzed on a Waters nanoAcquity using a 75 μm × 250 mm nanoAcquity UPLC column that was eluted at a flow rate of 300 nL/min as described in **Subheading 3.1.** The fast gradient in **Subheading 3.1** was used and peak detection was at 210 nm.

by simply decreasing the flowrate (while maintaining a constant gradient time program) eventually, the linear flow velocity on the column will be reduced to such an extent that optimal resolution will be lost ***(5–7)***. At this point, the column diameter needs to be decreased so that a more optimal linear flow velocity can be maintained at a lower flowrate. In general, the sensitivity of detection is increased as the wavelength is decreased with the practical limit in 0.05% TFA being about 210 nm. Finally, an important determinant of sensitivity (that is often overlooked) is the path length of the flow cell. For instance, an HP1090 equipped with a 0.6-cm path length cell provides (at the same flowrate) a threefold increase in sensitivity over that afforded by a Michrom UMA System equipped with a 0.2-cm path length cell. The ultra performance LC systems have addressed the sensitivity issue by concentrating the analytes into a lower volume and thereby, yielding UV peaks that are narrower and taller.

2. If the transit time (i.e., peak delay) between the detector and the fraction collector is too long, closely eluting peaks will be pooled together. The reason is that if a second peak is detected by the Isco Model 2150 Peak Separator while it is “counting down” the peak delay for the first peak, the two peaks will be pooled together. Although our experience is this phenomenon seldom occurs with a peak delay of 9 s (at a flowrate of 50 μL/min),

which corresponds to a "dead volume" of about 7.5 µL, it often occurs if the peak delay exceeds about 15 s.

3. The procedure we use to evaluate C-18 reverse-phase columns is to determine the relative number of peaks that are detected at a given slope sensitivity during the fractionation of an aliquot of a large-scale tryptic digest of transferrin ***(5–7)***.
4. The newer UPLC systems are capable of operating at up to 10,000 psi. This means that longer columns, which run at higher pressures at the same flow rates, can be run in order to increase the column peak capacity (i.e. the number of compounds that can be separated). Typical pressure on a 75 µm × 250 mm column running at 300 nl/min is 6100 psi.
5. For high-sensitivity work, the baseline may be "balanced" (after running the first blank run) by adding a small volume of 20% TFA (i.e., typically 10 to 100 µL) to either buffer A or B as needed ***(7)***. For the acetic and formic acid buffers, small amounts of these acids can also be added to flatten the baseline. In figure 1, buffer A contains 0.1% HCOOH while buffer B only 0.075% HCOOH. By decreasing the amount of HCOOH in buffer B, it was possible to flatten the baseline sufficiently so that a wavelength of 210 nm (which is more sensitive than 214nm) could be used.
6. Because filtering HPLC solvents may result in their contamination ***(7)***, we recommend they be made with HPLC-grade water and acetonitrile, and that they not be filtered prior to use.
7. Provided the fractions are tightly capped within a few hours of collection (to prevent loss of acetonitrile owing to evaporation), the acetonitrile is extremely effective at preventing microbial growth and peptide loss owing to adsorption. Under these conditions, we have often successfully sequenced peptide fractions that have been stored for longer than a year.
8. Provided that samples of both the modified and unmodified protein are available, comparative HPLC peptide mapping provides an extremely facile means of rapidly identifying peptides that contain posttranslational modifications. In the case of proteins that have been expressed in *E. coli*, the latter can often serve as the unmodified control, since relatively few posttranslational modifications occur in this organism. Certainly, the first attempt at comparative HPLC peptide mapping should be with enzymes, such as trypsin or lysyl endopeptidase, that have high specificity, and the digests should be separated using the acid/acetonitrile gradients. Although elution position (as detected by absorbance at 210 nm) provides a sensitive criterion to detect subtle alterations in structure, the value of comparative HPLC peptide mapping is further enhanced by multiwavelength monitoring and, especially, by online or off-line mass spectrometry of the resulting peptides. If comparative peptide mapping fails to reveal any significant changes, it is often worthwhile running the same digest in the pH 6.0 phosphate-buffered system (which may

not be compatible with on-line mass spectrometry). At this higher pH, some changes, such as deamidation of asparagine and glutamine, produce a larger effect on elution position than at pH 2.0, where ionization of the side-chain carboxyl groups would be suppressed. Another possible reason for failing to detect differences on comparative HPLC is that the peptide(s) containing the modifications are either too hydrophilic to bind or too hydrophobic to elute from reverse-phase supports. Hence, in addition to trying a different HPLC solvent system, another approach that may be taken to expand the capabilities of comparative HPLC peptide mapping is to try a different proteolytic enzyme, such as chymotrypsin or Protease V8. Finally, the failure to observe any difference on comparative HPLC peptide mapping may result from loss of the posttranslational modification during either the cleavage or the subsequent HPLC. Assignment of disulfide bonds is one example where this can be a problem in that disulfide interchange may occur during enzymatic cleavage, which is typically carried out at pH 8.0. This problem can be addressed by either going to shorter digestion times ***(9)*** or by carrying out the cleavage under acidic conditions, where disulfide interchange is less likely to occur. For this reason, pepsin (which is active in 5% formic acid) digests are often used for isolating disulfide bonded peptides. Providing that the control sample is reduced, comparative HPLC peptide mapping can be used to identify disulfide-linked peptides rapidly. If the sequence of the protein of interest is known, then comparison of the MALDI-MS spectra obtained before and after reduction of the disulfide-linked peptide can be used to identify the two peptides that are disulfide-bonded.

9. The purpose of the 2*M* urea is to minimize adsorptive losses on diluting the peptide fraction (which is accompanied by a 50% decrease in the acetonitrile concentration). We have found that provided the urea is made up (at least weekly) in NH_4HCO, no detectable NH_2-terminal blocking occurs as the result of cyanate formation.
10. The advantage of increasing the pH from 2.0, which is the approximate pH of the usual 0.05% TFA mobile phase, to pH 6.0 is that (as mentioned above) this change accentuates the separation of peptides based on their content of acidic amino acid residues. That is, since the P*Ka* of the acidic side chains of aspartic and glutamic acid is about 4, increasing the pH of the mobile phase from 2.0 to 6.0 results in ionization of their COOH side-chains. The increased charge that accompanies ionization greatly decreases retention of peptides on reverse-phase supports ***(2)***.

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Peptide Mapping by Two-Dimensional Thin-Layer Electrophoresis-Thin-Layer Chromatography

Ralph C. Judd

1. Introduction

The principle behind peptide mapping is straightforward: If two proteins have the same primary structures, then cleavage of each protein with a specific protease or chemical cleavage reagent will yield identical peptide fragments. However, if the proteins have different primary structures, and then the cleavage will generate unrelated peptides. The similarity or dissimilarity of the proteins' primary structure is reflected in the similarity or dissimilarity of the peptide fragments. Separation of peptides by 2-D thin-layer electrophoresis–thin-layer chromatography (2-D TLE-TLC) results in very high resolution of the peptides, making subtle comparisons possible. There are four phases to the 2-D TLE-TLC peptide mapping process:

1. Identification and purification of the proteins to be compared;
2. Radiolabeling of the proteins, and thus the peptide fragments, to minimize the quantity of protein required;
3. Cleavage of the proteins with specific endopeptidic reagents, either chemical or enzymatic; and
4. Separation and visualization of the peptide fragments for comparison.

Each step can be accomplished in different ways depending on the amount of protein available, the technologies available, and the needs of the researcher. Basic procedures that have proven reliable are presented for each phase. Because peptide mapping is empirical by nature, reaction times, reagent concentrations, and amounts of proteins and peptides may need to be altered to accommodate different research requirements.

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2. Materials

2.1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

See Chapter 21 for reagents and procedures for SDS-PAGE.

2.2. Electroblotting

1. Blotting chamber with cooling coil (e.g., Transblot chamber, Bio-Rad, Inc., Hercules, CA, or equivalent).
2. Power pack (e.g., EC 420, EC Apparatus Inc. [St. Petersburg, FL], or equivalent).
3. Nitrocellulose paper (NCP).
4. Polyvinylidene difluoride (PVDF) nylon membrane.
5. Ponceau S: 1–2 mL/100 mL H_2O.
6. Naphthol blue black (NBB): 1% in H_2O.
7. India ink (Pelikan, Hannover, Germany).
8. 0.05% Tween-20 in phosphate buffered saline (PBS), pH 7.4.
9. 20 m*M* phosphate buffer, pH 8.0: 89 mL 0.2 *M* Na_2HPO_4 stock, 11 mL 0.2*M* stock in 900 mL H_2O.

2.3. Radiolabeling

1. γ-Radiation detector.
2. Speed-Vac concentrator (Savant Inst. Inc., Farmingdale, NY) or any other drying system, such as heat lamps, warm air, and so forth, will suffice.
3. Carrier-free ^{125}I (*see* **Notes 1** and **2**).
4. 1,3,4,6-tetrachloro-3α,6α-glycouril (Iodogen). Iodogen-tubes are prepared by placing 10 µL of chloroform containing 1 mg/mL Iodogen in the bottom of 1.5-mL polypropylene microfuge tubes and allowing to air-dry. Iodogen tubes can be stored at –20°C for up to 6 mo.
5. PBS, pH 7.4 (any dilute, neutral buffer should work).
6. Dowex 1-X-8, 20–50 mesh, anion-exchange resin (Bio-Rad).
7. Twenty-four-well disposable microtiter plate.
8. Sephadex G-25 or G-50 (Pharmacia, Piscataway, NJ).
9. 15% Methanol in H_2O.
10. XAR-5 film (Kodak, Rochester, NY, or equivalent).
11. Lightening Plus intensifying screens (DuPont, Wilmington, DE, or equivalent).

2.4. Protein Cleavage

1. Enzymatic and chemical cleavage reagents and appropriate buffers as described in Chapter 94.
2. 88 or 70% Formic acid.
3. 50 m*M* NH_4HCO_3 adjusted to the appropriate pH with sodium hydroxide.
4. 50% Glacial acetic acid in H_2O.
5. Glacial acetic acid added to H_2O to bring pH to 3.0.

2.5. Peptide Separation

2.5.1. 2-D TLE-TLC

1. Forma 2095 refrigerated cooling bath (Forma Scientific, Marietta, OH) or equivalent.
2. Immersion TLE chamber (e.g., Savant TLE 20 electrophoresis chamber, or equivalent).
3. 1200-V Power pack.
4. Chromatography chambers.
5. "Varsol" (EC123, Savant, or equivalent).
6. 0.1-mm Mylar-backed cellulose sheets (E. Merck, MCB Reagents, Gibbstown, NJ, or equivalent).
7. TLE buffer: 2 L H_2O, 100 mL glacial acetic acid, 10 mL pyridine.
8. TLC buffer: 260 mL *n*-butanol, 200 mL pyridine, 160 mL H_2O, 40 mL glacial acetic acid.
9. H_2O containing Tyr, Ile, and Asp (1 mg/mL). These are 2-D TLC-TLE amino acid markers.
10. 1% Methyl green in H_2O (w/v).
11. Laboratory sprayer.
12. 0.25% Ninhydrin in acetone.
13. XAR-5 film (Kodak or equivalent).
14. Lightening Plus intensifying screens (DuPont or equivalent).

3. Methods

3.1. Protein Punfication

3.1.1. SDS-PAGE

Any protein purification procedure that results in 95–100% purity is suitable for peptide mapping. For analytical purposes, the discontinuous buffer, SDS-PAGE procedure is the best choice ***(1,2)***. SDS-PAGE provides apparent molecular-mass information, and the ability to probe SDS-PAGE-separated proteins by immunoblotting, helps ensure that the proper proteins are being studied.

If the proteins to be compared are abundant (>100 µg), peptide fragments can be visualized following 2-D TLE-TLC by ninhydrin staining. Much smaller amounts <0.05 µg) can be visualized by autoradiography if the proteins are extrinsically labeled with ^{125}I. Resolution of peptides increases as the amount of each peptide decreases. For these reasons, it is highly recommended that radiolabeled proteins be used ***(1,3–5)***. Proteins separated in SDS-PAGE gels can be labeled and cleaved directly in gel slices ***(3–7*** and *see* Chapter 99), but labeling and cleavage are much more efficient if the proteins are first electroblotted to NCP ***(4)***. Proteins can also be intrinsically or extrinsically labeled before SDS-PAGE separation ***(5)***. It is strongly recommended that even highly pure proteins be separated in SDS-PAGE gels and transferred to NCP because of the ease of labeling and cleavage using this system. Blotted proteins can be readily located by staining with Ponceau S in water (preferred), NBB in water, or India ink-0.05% Tween-20-PBS ***(8)***. Proteins of interest can then be excised, labeled using ^{125}I, and cleaved directly on the NCP ***(4)***. The peptides are released into the supernatant and can then be separated using 2-D TLE-TLC ***(4)***.

A single SDS-PAGE separation is often adequate to purify proteins for peptide mapping. Occasionally, a second separation may be required. Alternately, 2-D isoelectric focusing-SDS-PAGE can be used to purify proteins. If ^{125}I-labeling is used, a single protein band from a single lane of a 24-tooth comb is ample material for numerous separations of peptide fragments. Again, labeling and cleavage are greatly facilitated by electroblotting the protein to NCP.

3.1.1.1. Single SDS-PAGE Separation

1. Samples to be compared can be separated in individual lanes of an SDS-PAGE gel; "preparative" gels, where each sample is loaded over the entire stacking gel ***(4)***, may also be used.
2. After electrophoresis, fixation, Coomassie brilliant blue (CBB) staining, and destaining (*see* Chapter 21), excise the protein bands of interest for use in the "gel slice" methods described below. The preferred method is to electroblot the protein to NCP, at 20 V constant current, 0.6 A for 16 h in degassed 20 m*M* phosphate buffer, pH 8.0 ***(9)*** (*see* Chapters 58 and 59).
3. To stain the proteins on NCP, shake the NCP in Ponceau S for 15 min, then destain with H_2O, or shake in 0.1% NBB in H_2O for 1 h, and then destain with H_2O. If the proteins cannot be located by using these stains, place the NCP in 100 mL of 0.05% Tween-20 PBS, and mix for 1 h. Then add three drops of India ink and mix for another hour. Protein bands will be black and the background white (*see* **Note 3**).
4. Excise the protein band from the NCP (a 1 × 5-mm band is more than ample), and place the excised strip in a 1.5-mL microfuge tube. Wash with H_2O until no stain is released into the supernatant. The protein is now ready for labeling and cleavage (*see* **Note 4**).

3.1.1.2. Double SDS-PAGE Separation

1. Separate the samples in individual lanes of an SDS-PAGE gel or in "preparative" SDS-PAGE gels. Fix, stain with CBB, and then destain (*see* Chapter 21).
2. Excise the protein bands of interest. Soak the bands in 50% ethanol–50% stacking buffer (1 *M* Tris-HC1, pH 6.8) for 30 min to shrink the gel strip to facilitate loading onto a second SDS-PAGE gel.
3. Push the excised band into contact with the stacking gel of a second SDS-PAGE gel of a different acrylamide concentration (generally use high concentration in the first gel and lower concentration in the second gel).
4. Separate proteins in a second gel (CBB runs just behind the dye front). Stain or electroblot the proteins as in **Subheading 3.1.1.** The protein is now ready for labeling and cleavage.

3.2. Protein Labeling

Proteins can be intrinsically labeled by growing organisms in the presence of a uniform mixture of ^{14}C-amino acids ***(5)***, but this is quite expensive. Intrinsic labeling with individual amino acids, such as ^{35}S-Met or ^{35}S-Cys, will not work, since many peptide fragments will not be labeled. Iodination with ^{125}I is inexpensive and reproducible. Iodinated peptides are readily visualized by autoradiography ***(1, 3, 4)***. Comparative cleavages of a 40,000-Dalton protein intrinsically labeled with ^{14}C-amino acids extrinsically labeled with ^{125}I showed that 61 of 66 α-chymotryptic peptides were labeled with ^{125}I, whereas all 22 *Staphylococcus aureus* V8 protease-generated peptides were labeled with ^{125}I, demonstrating the effectiveness of radioiodination ***(10)***. This demonstrates that tyrosine (Tyr) is not the only amino acid labeled using this procedure.

Iodination mediated by chloramine-T (CT) ***(11)*** produces extremely high specific activities, but the procedure requires an extra step to remove the CT and can cleave some proteins at tryptophan residues ***(12)***. This can be beneficial since it is specific and increases the number of peptides, thus increasing the sensitivity of the procedure (*see* **ref. *13***) for peptide maps of CT- vs Iodogen-labeled proteins). Unfortunately, small peptides generated by CT cleavage, followed by a second enzymatic or chemical cleavage, can be lost during the removal of the CT and unbound ^{125}I.

The 1,3,4,6-tetrachloro-3α,6α-glycouril (Iodogen) ***(13)*** procedure, where the oxidizing agent is bound to the reaction vessel, does not damage the protein and produces high specific activities. Aspiration of the reaction mixture stops the iodination and separates the protein from the oxidant in a single step. For these reasons, Iodogen-mediated labeling is the preferred method for radioiodination. (Radioemission of ^{125}I will be expressed as counts per minute [cpm]. This assumes a detector efficiency of 70%. If detector efficiency varies, multiply the cpm presented here by 1.43 to determine decays per minute (dpm), and then multiply the dpm by the efficiency of your detector.)

3.2.1. NCP Strip (Preferred Method)

1. Put the protein-containing NCP strip in an Iodogen-coated (10 µg) microfuge tube.
2. Add 50–100 µL PBS, pH 7.4 (any dilute, neutral buffer should work) and 50–100 µCi ^{125}I (as NaI, carrier-free, 25 µCi/µL) (*see* **Notes 1** and **2**).
3. Incubate at room temperature for 1 h. Aspirate the supernatant. **(Caution: supernatant is radioactive).**
4. Place the NCP strip in a fresh microfuge tube and wash three to five times with 1.5 mL H_2O (radioactivity released should stabilize at <10,000 cpm/wash).
5. The protein on the NCP strip is now ready for cleavage (*see* **Note 5**).

3.2.2. Gel Slice

1. Dry the gel slice containing protein using a Speed-Vac concentrator or other drying system, such as heat lamps, warm air, and so forth.
2. Put the slice in an Iodogen-coated (10-µg) microfuge tube.
3. Add 100 µL of PBS, pH 7.4 (any dilute, neutral buffer should work) plus 50–100 µCi ^{125}I (as NaI, carrier-free, 25 µCi/µL) (*see* **Notes 1** and **2**).

4. Incubate at room temperature for 1 h. Aspirate the supernatant. **(Caution: supernatant is radioactive)**.
5. Remove the gel slice and soak for 0.5 to 1 h in 1.5 mL of H_2O. Repeat three times.
6. Place 0.5 g Dowex 1-X-8, 20–50 mesh, anion-exchange resin and 1.5 mL of 15% methanol in H_2O in the wells of a 24-well microtiter plate.
7. Add the iodinated gel slice to a well with anion-exchange resin and incubate at room temperature for 16 h. The resin binds unreacted iodine, becoming **extremely** radioactive.
8. Remove the gel slice from the resin, and soak it in 1.5 mL of H_2O. Repeat several times, and dry the gel slice. The protein is now ready for cleavage (*see* **Note 6**).

3.2.3. Lyophilized/Soluble Protein

1. Suspend up to 1 mg/mL of protein in 100–200 µL of PBS, pH 7.4 (any dilute, neutral buffer should work), in a 1.5-mL microfuge tube containing 10 µg Iodogen.
2. Add 100–200 µCi ^{125}I (as NaI, carrier-free, 25 µCi/µL) (*see* **Notes 1** and **2**).
3. Incubate on ice for 1 h.
4. Remove the protein-containing supernatant and separate the protein from salts and unbound iodine by the following methods:
   a. (Preferred method) Separate on a Sephadex G-25 or G-50 desalting column using H_2O as the eluant and lyophilize.
   b. (Relatively easy) Solubilize the sample in 2X sample buffer (10–20 µg/lane), and separate in an SDS-PAGE gel. Stop the electrophoresis before the ion front reaches the bottom of the gel and cut the gel just above the dye front. Unbound iodine will be in this portion of the gel. Either fix, stain, and destain the gel to locate the protein band, or electroblot onto NCP and locate the protein by Ponceau S. NBB, or India ink staining (*see* **Subheading 3.1.1.1, step 3**). Excise the protein band from the gel or NCP.
   c. (Excellent if available) Separate the protein using reverse-phase or molecular-exclusion HPLC columns, and then dialyze and lyophilize.
   d. (Least preferred) Dialysis, followed by lyophilization, can be used, but it produces excessive radioactive liquid waste.
5. The protein is now ready for cleavage.

3.3. Protein Cleavage

The use of cleavage reagents (e.g., α-chymotrypsin) or combinations of cleavage reagents, which generate many fragments, tends to accentuate differences in primary structure, whereas cleavage reagents that produce small numbers of fragments (e.g., V8 protease, thermolysin, CNBr, BNPS-skatole) emphasize similarities in primary structure. Enzymatic reagents are often easiest to use, safest, and most reliable, but they can interfere with results, since they are themselves proteins. Chemical reagents are also easy to use and reliable, but can be toxic, requiring careful handling (*see* **Note 7**). Several practical cleavage reagents are presented in **Table 1**.

Table 1
Cleavage Reagents

Reagent	Site of cleavage	Buffer
Chemical		
Cyanogen bromide[a,d]	Carboxy side of Met	70% formate in H_2O
BNPS-skatole[a] (-bromo-methyl-2-(nitrophenylmercapto)-3H-indole)	Carboxy side of Trp	50% H_2O–50% glacial acetic acid
Formic acid	Between Asp and Pro	88% in H_2O
Chloramine T[b]	Carboxy side of Trp	H_2O
Enzymatic[c]		
α-chymotrypsin	Carboxy side of Tyr, Trp, Phe, Leu	50 m*M* NH_4HCO_3, pH 8.5
Pepsin A	Amino side of Phe>Leu	Acetate- H_2O, pH 3.0
Thermolysin	Carboxy side of Leu>Phe	50 m*M* NH_4HCO_3, pH 7.85
Trypsin	Carboxy side of Arg, Lys	50 m*M* NH_4HCO_3, pH 8.5
V8 protease	Carboxy side of Glu, Asp, or carboxy side of Glu	50 m*M* NH_4HCO_3, pH 8.5 pH 6–7 (H_2O)

[a] Cyanogen bromide and BNPS-skatole are used at 1 mg/mL. Room temperature incubation should proceed for 24–48 h under nitrogen in the dark.
[b] Chloramine T is used at 10 mg/mL in H_2O.
[c] All enzymes are used at 1 mg/mL in the appropriate buffer.
[d] **Caution:** CNBr is extremely toxic—handle with care in chemical hood.

Volatile buffers must be used when using 2-D TLE-TLC peptide separation, since this system is negatively affected by salts. For formate and CNBr cleavages, the acid is diluted in H_2O to 88 or 70%, respectively. BNPS-skatole works well in 50% glacial acetic acid-50% H_2O. Ammonium bicarbonate (50 m*M*), adjusted to the appropriate pH with sodium hydroxide, is excellent for enzymes requiring weak base environments (trypsin, α-chymotrypsin, thermolysin, V8 protease). The acid peptidase, Pepsin A, is active in H_2O adjusted to pH 3.0 with glacial acetic acid.

3.3.1. Protein on NCP Strip

1 Put the NCP strip containing the radiolabeled protein in a 1.5-mL microfuge tube, and measure the radioemission using a γ-radiation detector.

2. Add 90 μL of the appropriate buffer and 10 μL of chemical or enzymatic cleavage reagent in buffer (1 mg/mL) to the NCP strip.
3. Incubate with shaking at 37°C for 4 h (for enzymes) or at room temperature for 24–48 h in dark under nitrogen (for chemical reagents).
4. Aspirate the peptide-containing supernatant, and count the NCP strip and supernatant. Enzymes should release 60–70% of counts in the slice into the supernatant; CNBr should release >80%, BNPS-skatole rarely releases more than 50% (*see* **Notes 8** and **9**).
5. Completely dry the supernatant in a Speed- Vac, and wash the sample at least four times by adding 50 μL of H_2O, vortexing, and redrying in a Speed-Vac. Alternate drying systems will work.
6. The sample is now ready for peptide separation (*see* **Note 10**).

3.3.2. Protein in Gel Slice

1. Put the dry gel slice containing the radiolabeled protein in a 1.5-mL microfuge tube and measure the radioemission using a γ-radiation detector.
2. Add 10 μL of cleavage reagent in buffer (1 mg/mL) directly to the dry gel slice. Allow slice to absorb cleavage reagent, and then add 90 μL of appropriate buffer.
3. Continue as from **step 3, Subheading 3.3.1**. Release of peptides into the supernatant will be less efficient than with the NCP strip.
4. The sample is now ready for peptide separation (*see* **Note 10**).

3.3.3. Lyophilized/SolubleProteins

1. Rehydrate the lyophilized radiolabeled proteins in the appropriate buffer at 1 mg/mL (less concentrated samples can be used successfully).
2. Add up to 25 μL of the appropriate cleavage reagent (1 mg/mL) to 25 μL of suspended protein.
3. Continue as from **step 3, Subheading 3.3.1.,** except there is no strip to count.
4. The sample is now ready for peptide separation. Be aware that the sample will usually contain uncleared protein along with the peptide fragments (*see* **Note 10**).

3.4. 2-D TLE-TLC Peptide Separation

It is strongly recommended that iodinated samples be used in the 2-D TLE-TLC system. The technique described is precise enough that peptide maps can be overlaid to facilitate comparisons. Flat-bed electrophoresis can be used, but systems that cool by immersion of the thin-layer sheet in an inert coolant, such as "varsol" (such as the Savant TLE 20 electrophoresis chamber or equivalent), yield superior results. Cooling should be supplied by as large a refrigerated bath as possible, such as the Forma 2095 refrigerated cooling bath. Extra cooling coils, made by bending 1/4 in. aluminum tubing, are helpful. Peptides migrate based on charge, which is a function of pH, in an electric field. The buffer pH is a function of temperature. Therefore, maintenance of the running buffer temperature is crucial. Inconsistent cooling results in inconsistent peptide migration. For best results, use only 0.1 mm Mylar backed cellulose sheets of E. Merck. Run two or three peptide maps per 20 × 20-cm sheet. If necessary, increased resolution can be obtained by running one sample/sheet and increasing running times (*see* **Note 11**).

1. Set the cooling bath at 8.5°C to keep the electrophoresis tank at 10–13.5°C. The temperature of the cooling tank should not increase more than 1.5°C during a run.
2. Rehydrate the peptide sample to 10^5 cpm/μL in H_2O containing Tyr, Ile, and Asp (1 mg/mL) as amino acid markers.
3. For two samples/run: draw a line down the center of the back of the sheet with a laboratory marker parallel to the machine lines (they can be subtle, but always electrophorese parallel to these lines). Mark two spots 2.5 cm from the end of the sheet and 1.5 cm from center line on the back of the sheet to indicate where to load samples. When the sheet is turned with the cellulose facing up, the marks will show through. For three samples/run: draw two lines of the back of the sheet (parallel to machine lines) 6.7 and 13.4 cm from left edge of sheet and mark three spots, each 8 cm from the end of the plate and 1 cm to the right of the left edge and each line (*see* **Note 12**).
4. Use a graduated, 1–5 μL capillary pipette to spot 2 μL (~ 2 × 10^5 cpm) if two samples are used, or 1.5 μL (~ 1.5 × 10^5 cpm) if three samples are used, 0.5 μL at a time (dry spot with hair-dryer each time) to one mark on the sheet. Repeat for each sample on the other mark(s). More sample can be run, but resolution will decrease. To verify proper electrophoresis, spot 1 μL of 1% methyl green on the center line. The methyl green should migrate rapidly toward the cathode in a straight line. Veering indicates a problem.
5. Spray the plate with TLE buffer using a laboratory sprayer. Do not over wet. Remove any standing buffer with one paper towel. Always blot TLE plates in exactly the same manner.
6. Place in the electrophoresis chamber with the samples toward the anode. Run the electrophoresis at 1200 V (about 20 W and 20 mA) for 45 min (two samples/run) or 31 min (three samples/run).
7. Remove the sheet from the chamber, and immediately dry with a hair-dryer. The "varsol" will dry first, and then the buffer. Cut along the lines on the back of the sheet. Score the cellulose 0.5 cm down from top edge of each piece (bottom is the edge closest to the sample) to form a moat.
8. Place the sheets in the chromatography chamber so that chromatography can proceed perpendicularly to the electrophoresis. The TLC buffer should be about 0.5-cm deep. Chromatograph until the buffer reaches the moat. Remove and dry with a hair-dryer (best done in hood).
9. Spray the sheet with 0.25% ninhydrin in acetone (do not saturate) and dry with a hair-dryer to locate amino acid markers. Ninhydrin can also be used to locate peptides if larger amounts of sample are separated (10–100 μg). Be sure to run enzyme controls to distinguish sample from enzyme. Markers should migrate identically in all separations.
10. Overlay the sheets with X-ray film, place Lightening Plus intensifying screen over film, and place in cassette. Expose for 16–24 h at –70°C or expose film without a screen for about 4 d at room temperature. Develop the film (*see* **Note 13**).

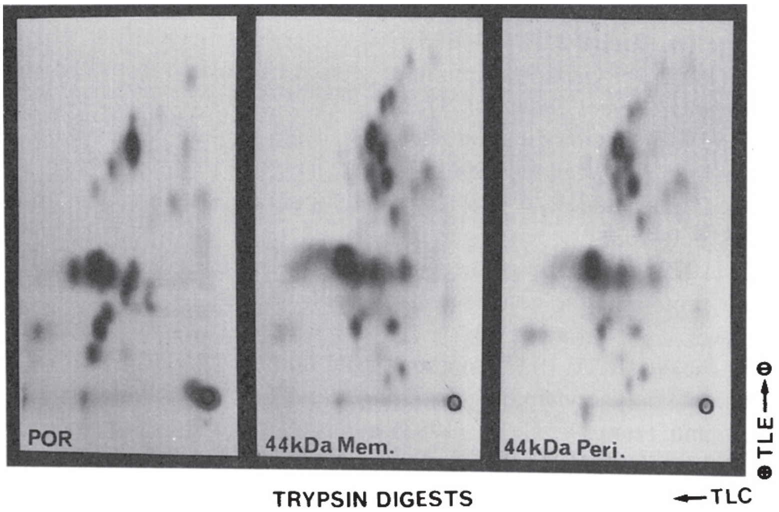

Fig. 1. Example of peptides separated by 2-D TLE-TLC. Proteins were radiolabeled on NCP strips as described in **Subheading 3.2.1.** and cleaved with trypsin as described in **Subheading 3.3.1.** The peptides (1×10^5 cpm) were spotted on a thin-layer cellulose sheet and subjected to 2-D TLE-TLC as described in **Subheading 3.4.** The origin (O) is at the lower right of each map. TLE-direction of thin- layer electrophoresis; TLC—direction of thin-layer chromatography. The ^{125}I-labeled peptides were visualized by autoradiography.

Figure 1 is presented to demonstrate the separation of peptides generated by cleavage with trypin by 2-D TLE-TLC. These peptide maps indicate that the porin protein (POR) of *Neisseria gonorrhoeae* is structurally unrelated to the 44-kDa proteins, whereas the 44-kDa protein from the sarkosyl insoluble (membrane) extract (44-kDa Mem.) is structurally indistinguishable from the 44-kDa protein from the periplasmic extract (44-kDa Peri.). Note the high resolution of the peptide fragments using this technique.

4. Notes

1. Regardless of the labeling procedure, never use ^{125}I that is over one half-life (60 d) old. Do not increase the amount of older ^{125}I to bring up the activity; it does not work.
2. Never use carrier-free ^{125}I in acid buffers. The iodine becomes volatile and could be inhaled.
3. Ponceau S can be completely removed with H_2O, but NBB or India ink cannot.
4. Do not compare proteins stained with Ponceau S with those stained with NBB or India ink. Use the same staining procedure for all proteins to be compared.

5. It is common to have between 3 × 10^6 and 6 × 10^6 cpm for a strip 1 × 5 mm. This provides enough material to run 15–30 peptide maps/strip.
6. It is common to have between 2 × 10^6 and 4 × 10^6 cpm in a gel slice (1 × 5 mm). This provides enough material to run 10–20 peptide maps/slice.
7. Chemical cleavage reagents are preferred when peptides are not radiolabeled since enzymes cleave themselves, resulting in confusing data. **CNBr is extremely toxic—handle with great care in a chemical hood**.
8. Repeated digestions will release about the same percentage of counts. Generally, one or two digestions are adequate. Only 1 × 10^5 cpm are necessary to produce a peptide map.
9. If >95% of counts are released in the first enzyme digestion, there may be excess, unbound iodine left in sample. This could cause serious problems, since the enzyme may become labeled. The resultant peptide maps will all be identical maps of the enzyme and not your sample.
10. Peptides prepared in this manner can also be separated by high-performance liquid chromatography (*see* Chapter 102).
11. CNBr should not be used to generate peptides for use in 2-D TLE-TLC, since it produces very hydrophobic peptides, which tend to compress at the top of the chromatogram. CNBr is excellent for generating peptides to be separated by SDS-PAGE or HPLC.
12. Always spot sample to be compared the same distance from the anode (positive) terminal, since peptides migrate more rapidly close to the anode and more slowly far from the anode.
13. Migration of peptides should be consistent enough to overlay peptide maps directly for comparisons. Coordinates of amino acid markers and peptides can be determined and used to compare migration. The labeling procedures described here are precise enough to use emission intensities as a criterion for comparison.

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Peptide Mapping by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Ralph C. Judd

1. Introduction

The comparison of the primary structure of proteins is an important facet in the characterization of families of proteins from the same organism, similar proteins from different organisms, and cloned gene products. There are many methods available to establish the sequence similarities of proteins. A relatively uncomplicated approach is to compare the peptide fragments of proteins generated by enzymatic or chemical cleavage, i.e., peptide mapping. The similarity or dissimilarity of the resultant peptides reflects the similarity or dissimilarity of the parent proteins.

One reliable method of peptide mapping is to separate peptide fragments by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Comparison of the separation patterns reveals the structural relationship of the proteins. Moderate separation of peptides can be accomplished using this procedure. The technique, first described by Cleveland et al. ***(1)***, is simple, inexpensive, requires no special equipment, and can be combined with Western blotting to locate epitopes (i.e., epitope mapping ***[1–3]***) or blotting to nylon membranes for microsequencing ***(4)***. Microgram amounts of peptide fragments can be visualized by in-gel staining, making this system fairly sensitive. Sensitivity can be greatly enhanced by radiolabeling (*see* Chapter 102).

2. Materials

2.1. SDS-PAGE

1. SDS-PAGE gel apparatus and power pack (e.g., EC 500, EC Apparatus, Inc., St. Petersburg, FL, or equivalent).

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2. SDS-PAGE Solubilization buffer: 2 mL 10% SDS (w/v) in H_2O, 1.0 mL glycerol, 0.625 mL 1 *M* Tris-HCl, pH 6.8, 6 mL H_2O, bromophenol blue to color.
3. Enzyme buffer for Cleveland et al. ***(1)*** "in-gel" digestion: 1% SDS, 1 m*M* EDTA, 1% glycerol, 0.1 *M* Tris-HCl, pH 6.8.
4. All buffers and acrylamide solutions necessary for running SDS-PAGE (*see* Chapter 21).
5. Ethanol: 1 *M* Tris-HCl, pH 6.8 (50:50; [v/v]).
6. Fixer/destainer: 7% acetic acid, 25% isopropanol in H_2O (v/v/v).
7. Coomassie brilliant blue: 1% in fixer/destainer (w/v).
8. Mol-wt markers, e.g., low-mol-wt kit (Bio-Rad, Hercules, CA, or equivalent) or peptide mol-wt markers (Pharmacia, Piscataway, NJ, or equivalent).

2.2. Electroblotting (for Epitope or Sequence Analyses)

1. Blotting chamber with cooling coil (e.g., Transblot chamber, Bio-Rad, Inc. or equivalent).
2. Power pack (e.g., EC 420, EC Apparatus, Inc. or equivalent).
3. Nitrocellulose paper (NCP).
4. Polyvinylidene difluoride (PVDF) nylon membrane.
5. Ponceau S: 1–2 mL/100 mL H_2O.
6. 1% Naphthol blue black (NBB) in H_2O (w/v).
7. 0.05% Tween-20 in phosphate-buffered saline (PBS), pH 7.4.
8. India ink, three drops in 0.05% Tween-20 in PBS, pH 7.4.
9. 20 m*M* phosphate buffer, pH 8.0: 89 mL 0.2 *M* Na_2HPO_4 stock, 11 mL 0.2 *M* NaH_2PO_4 stock in 900 mL H_2O.

3. Methods

3.1. Protein Purification

Purification of the proteins to be compared is the first step in peptide mapping. Any protein purification procedure that results in 95–100% purity is suitable for peptide mapping. For analytical purposes, the discontinuous buffer, SDS-PAGE procedure is often the best choice (Chapter 21 and **refs.** ***2*** and ***5***). The advantages of SDS-PAGE are: Its resolving power generally can bring proteins to adequate purity in one separation, whereas a second SDS-PAGE separation almost always provides the required purity for even the most difficult proteins; the simple reliability of the procedure; both soluble and insoluble proteins can be purified; apparent molecular-mass information; and the ability to probe SDS-PAGE-separated proteins by immunoblotting help ensure that the proper proteins are being studied. If even greater separation is required, 2D isoelectric focusing-SDS-PAGE can be used.

Proteins separated in SDS-PAGE gels can be cleaved directly in gel slices ***(6)***. However, cleavage is more efficient if the proteins are first electroblotted to NCP ***(3)***. If required, proteins can also be intrinsically or extrinsically labeled before SDS-PAGE separation ***(6).***

3.2. Protein Cleavage

Cleavages of purified proteins can be accomplished in the stacking gel with the resultant peptides separated directly in the separating gel, or they can be performed prior to loading the gel (preferred). Several lanes, with increasing incubation time or increasing concentration of enzyme in each lane, should be run to determine optimal proteolysis conditions. Standard Laemmli SDS-PAGE ***(5)*** system is able to resolve peptide fragments >3000 Dalton. Smaller peptides are best separated in the tricine gel system of Schagger and von Jagow ***(7)*** (*see also* Chapter 23). The methods described in Chapter 97, **Subheading 3.3** can be used to generate peptides. Cleavage technique varies slightly depending on the form of the purified proteins to be compared.

3.2.1. In-Gel Cleavage/Separation

3.2.1.1. LYOPHILIZED/SOLUBLE PROTEIN

1. Boil the purified protein (~1 mg/mL) for 5 min in enzyme buffer for Cleveland "in-gel" digestion.
2. Load 10–30 µL (10–30 µg) of each protein to be compared into three separate wells of an SDS-PAGE gel (*see* **Note 1**).
3. Overlay samples of each protein with 0.005, 0.05, and 0.5 µg enzyme in 10–20 µL of Cleveland "in-gel" digestion buffer to separate wells. V8 protease (endoproteinase Glu-C) works very well in this system (*see* **Note 2**). SDS hinders the activity of trypsin, α-chymotrypsin, and themolysin, so cleavage with these enzymes may be very slow. In-gel cleavage with chemical reagents is not generally recommended, since they can be inefficient in neutral, oxygenated environments. Gently fill wells and top chamber with running buffer.
4. Subject the samples to electrophoresis until the dye reaches the bottom of the stacking gel. Turn off the power, and incubate for 2 h at 37°C to allow the enzyme to digest the protein partially. Following incubation, continue electrophoresis until the dye reaches the bottom of the gel. Fix, stain, and destain, or electroblot onto NCP for immunoanalysis (*see* **Note 3**).

3.2.1.2. PROTEIN IN-GEL-SLICE

Peptides of proteins purified by SDS-PAGE usually retain adequate SDS to migrate into a second gel without further treatment.

1. Run SDS-PAGE gels, fix, stain with Coomassie brilliant blue, and destain.
2. Excise protein bands to be compared with a razor blade.
3. Soak excised gel slices containing proteins to be compared in ethanol–1 *M* Tris-HCl, pH 6.8, for 30 min to shrink the gel, making loading on the second gel easier. Place the gel slices into the wells of a second gel.
4. Continue from **step 3, Subheading 3.2.1.1**.

3.2.1.3. PROTEIN ON NCP STRIP

1. Run SDS-PAGE gels, and electroblot to NCP.
2. Stain blot with Ponceau S, NBB, or block with 0.05% Tween-20 in PBS for 30 min, and then add three drops India ink (*see* **Note 4**).
3. Excise protein bands to be compared with a razor blade.
4. Push NCP strips to bottom of the wells of a second SDS-PAGE gel.
5. Continue from **step 3, Subheading 3.2.1.1**.

Once the separation conditions, protein concentrations, and enzyme concentrations have been established, a single digestion lane for each sample can be used for comparative purposes.

3.2.2. Protein Cleavage Followed by SDS-PAGE

It is often preferable to cleave the proteins to be compared prior to loading onto an SDS-PAGE gel for separation. This generally gives more complete, reproducible cleavage and allows for the use of chemical cleavage reagents not suitable for in-gel cleavages.

3.2.2.1. LYOPHILIZED/SOLUBLE PROTEIN

1. Rehydrate the lyophilized proteins in the appropriate buffer at 1 mg/mL (less concentrated samples can be used successfully). Soluble proteins may need to be dialyzed against the proper buffer.
2. Place 10–30 µL (10–30 µg) of each protein to be compared in 1.5-mL microfuge tubes.
3. Add up to 25 µL of the appropriate chemical cleavage reagent (1 mg/mL) to suspended protein. For enzymes, use only as much enzyme as needed to achieve complete digestion (1:50 enzyme to sample maximum) (*see* **Note 5**).
4. Incubate with shaking at 37°C for 4 h (for enzymes) or at room temperature for 24–48 h in dark under nitrogen (for chemical reagents).
5. Add equal volume of 2X SDS-PAGE solubilization buffer and boil for 5 min (*see* **Note 6**).
6. Load entire sample on SDS-PAGE gel and proceed with electrophoresis.

3.2.2.2. PROTEIN IN-GEL SLICE

1. Run SDS-PAGE gels, fix, stain with Coomassie brilliant blue, and destain.
2. Excise protein bands to be compared with a razor blade.
3. Dry gel slices containing proteins using a Speed-Vac concentrator or other drying system, such as heat lamps, warm air, and so forth.
4. Put the dry gel slice containing the protein in a 1.5-mL microfuge tube.
5. Add up to 10 µL of the appropriate chemical cleavage reagent (1 mg/mL) directly to the gel slice protein, and then and 90 µL of appropriate buffer. For enzymes, use only as much enzyme as needed to achieve complete digestion (1:50 enzyme to sample maximum) (*see* **Note 5**).
6. Incubate with shaking at 37°C for 4 h (for enzymes) or at room temperature for 24–48 h in dark under nitrogen (for chemical reagents).

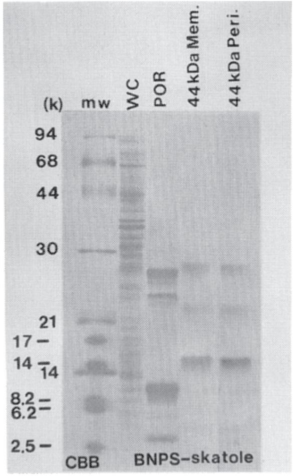

Fig. 1. Example of peptides separated in an SDS-PAGE gel. Whose cells (WC), a sarkosyl insoluble pellet, and a periplasmic extract of *Neisseria gonorrhoeae* were separated in "preparative" 15% SDS-PAGE gels and blotted to NCP as described in Chapter 103, **Subheading 3.1.1**. The 37,000-Dalton major outer membrane protein (POR) and two 44,000-Dalton (44-kDa) proteins, one isolated from a sarkosyl insoluble (membrane) extract (44-kDa Mem.), and the other isolated from a periplasmic extract (44-kDa Peri.), were located on the NCP by Ponceau S staining, excised, and cleaved with BNPS-skatole as described in Chapter 96, **Subheading 3.3.1**. Approximately 30 μg of peptides of each protein were solubilized and separated in an SDS-PAGE gel along with whole-cells (WC), Bio-Rad low-mol-wt markers, and Pharmacia peptide mol-wt markers (mw) (expressed in thousands of Dalton [k]). The gel was stained with Coomassie brilliant blue (CBB) to visualize peptides.

7. Aspirate peptide-containing supernatant.
8. Completely dry-down the supernatant in a Speed-Vac, and wash the sample several times by adding 50 μL of H_2O, vortexing, and redrying in a Speed-Vac. Alternate drying systems will work.
9. Add 10–20 μL SDS-PAGE solubilizing solution to samples and boil for 5 min (*see* **Note 6**).
10. Load samples onto SDS-PAGE gel and proceed with electrophoresis.

3.2.2.3. Protein on NCP Strip

1. Run SDS-PAGE gels, and electroblot to NCP.
2. Stain blot with Ponceau S, NBB, or block with 0.05% Tween-20 in PBS for 30 min, and then add three drops India ink (*see* **Note 3**).
3. Excise protein bands to be compared with a razor blade.
4. Put the NCP strip containing the protein in a 1.5-mL microfuge tube.
5. Continue from **step 5, Subheading 3.2.2.1**.

Figure 1 is presented to demonstrate the separation of peptides generated by cleavage with BNPS-skatole in an SDS-PAGE gel. These peptide maps indicate that the porin protein (POR) was structurally unrelated to the 44-kDa proteins, whereas the 44-kDa proteins from a sarkosyl insoluble (membrane) extract (44-kDa Mem.) and a periplasmic extract (44-kDa Peri.) appeared to have similar primary structures.

4. Notes

1. Between 5×10^4 cpm and 10^5 cpm should be loaded in each lane if radioiodinated samples are to be used. Autoradiography should be performed on unfixed gels. Fixation and staining may wash out small peptides. Place gel in a plastic bag and overlay with XAR-5 film, place in a cassette with a Lightening Plus intensifying screen, and expose for 16 h at −70°C.
2. Control lanes containing only enzyme must be run to distinguish enzyme bands from sample bands.
3. PVDF nylon membrane is preferable to NCP when blotting small peptides.
4. Do not compare proteins stained with Ponceau S with those stained with NBB or India ink. Use the same staining procedure for all proteins to be compared.
5. To ensure complete digestion, it is advisable to incubate the samples for increasing periods of time or to digest with increasing amounts of enzyme. Once optimal conditions are established, a single incubation time and enzyme concentration can be used.
6. It is often advisable to solubilize protein in SDS prior to cleavage, since some peptides do not bind SDS well.

Acknowledgments

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Peptide Mapping for Protein Characterization

Peter Højrup

1. Introduction

Analysis of intact proteins is hampered by the fact that even when using high resolution techniques, like electrospray mass spectrometry; you will only be able to tell whether the protein has the expected mass. If you find a deviation from the expected you will in most cases be left wondering where (and perhaps what) the difference is. Recent methods, such as top-down sequencing ***(1,2)***, can give you invaluable information on terminal trimming or even simple posttranslational modifications, but generally works best for "simple" changes to the protein. Observations on intact proteins are compounded when using low-resolution techniques like gel filtration or SDS gel electrophoresis. Although 2-D gel electrophoresis is able to show a single charge difference, in the absence of additional information you are still left wondering where and what the differences are.

Performing Edman degradation on an intact protein will in most cases yield enough information for you to identify a protein and perhaps enable the construction of oligonucleotide probes, but many proteins are N-terminally blocked, and the need to purify enough protein is often prohibitive.

The solution is to perform peptide mapping. This approach is essential for the total characterization of a protein, and the approach also enables quick identification of a protein (if present in a protein database) and to locate differences between proteins.

The steps involved in peptide mapping can be listed as:

1. Purify the protein. Depending on the objective of the peptide mapping, it may be sufficient with a single spot from a 2-D gel, or you may have to purify several microgram of material using traditional protein chemical methods.

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2. Cleave the protein using a suitable proteolytic enzyme or by chemical means. A software tool like GPMAW (*see* **Note 1**) greatly facilitates the selection of cleavage method and analysis of the resulting peptides.
3. Fractionate the resulting peptides, generally by reversed phase HPLC, but other methods may be applicable depending on the final method of analysis.
4. Analyze the purified peptide using mass spectrometry, Edman degradation, or amino acid analysis.

1.1. Peptide Mapping

Peptide mapping is usually carried out for two quite different reasons:

1.1.1. Protein Identification

Currently the most sensitive identification of an unknown protein is mass spectrometric peptide mapping (peptide mass fingerprinting, PMF). Using the rapidly expanding protein databases based on nucleotide sequencing, it is possible to identify a protein based on the molecular masses of its constituent (tryptic) peptides if they are determined with sufficient precision. The beauty of the method is that it is not necessary to determine the mass of all peptides; usually 6-7 peptides are sufficient for accurate protein identification. As it is not necessary to separate the peptides before analysis, the method is very sensitive; with routine determination of proteins in the low nanogram range (e.g. a single silver stained spot from an SDS gel).

Two alternative approaches are liquid chromatography coupled to electrospray mass spectrometry (LC-MS/MS) and Edman degradation. Both methods will generate a peptide sequence thus making it possible to do a homology search in case the protein is not present in the database. However, while the sensitivity of LC-MS/MS approaches that of MALDI-MS, the sensitivity of Edman degradation is 2-3 orders of magnitudes lower and further needs a purification step prior to analysis. The topic of LC-MS/MS is beyond the scope of this chapter and will not be treated further. For an introduction please see ***ref. (3)***.

1.1.2. Obtaining the Maximum Sequence Coverage

Unlike protein identification where even a limited number of peptides are able to identify the protein, you will try to cover the whole sequence when comparing proteins for identity/similarity or when performing complete chemical characterization.

1.1.2.1. COMPARISON OF PROTEINS

Comparison of two proteins is done by performing an identical enzymatic digest on each protein followed by identification of each fragment. In some cases it may be sufficient to perform mass spectrometric mapping of the peptide mixture, but in most cases you will not be able to identify sufficient fragments to cover the whole sequence. You will thus have to isolate each fragment by HPLC and perform peptide identification. Due to the high resolution of reversed phase HPLC it will in many cases be sufficient to compare the two chromatograms carefully in order to identify differences. When comparing proteins you should note that choosing an enzyme, which generates long peptide fragments, tends to emphasis similarities while short peptide fragments tends to emphasize differences (*see* **Note 2**).

1.1.2.2. Determine the Exact Chemical Structure of a Protein

Even though the number of known protein sequences increases exponentially over time through nucleotide sequence analysis, and you are thus likely to find your protein to be already known, the result of these analyses is the 'naked' amino acid sequence of the protein. However, the vast majority of proteins are post-translationally modified after synthesis, resulting in a structure that deviates from the nucleotide-derived sequence. The preferred strategy to solve the actual protein structure is to perform peptide mapping followed by an appropriate analytical techniques like mass spectrometry, Edman degradation and/or amino acid analysis. Here you always aim for total sequence coverage, and in order to obtain this you will in most cases have to perform at least two separate enzymatic digests.

The amount of material needed for maximum sequence coverage analysis is usually much higher than for PMF analysis, usually in the low to middle microgram range. In special cases where you search only for specific modifications using specific extraction of modified peptides (e.g. purification of phosphorylated peptides using titanium oxide), the sensitivity approach is similar to that of PMF ***(4)***.

2. Materials

2.1. Peptide Mapping for Protein Identification

1. Trypsin (modified trypsin, Promega,). This is most easily handled in aliquots of 1 µg trypsin in 10 µL 0.01 N HCl per tube at –20 °C. Dilute with 70 µL 50 m*M* ammonium bicarbonate before use (final concentration 12.5 ng /µL). Remember always to use UHQ (ultrahigh quality) water.
2. 50 m*M* ammonium bicarbonate. You can store aliquots of 100 m*M* ammonium bicarbonate at –20 °C, it is not necessary to adjust the pH. Dilute 1 : 1 for use.
3. Neat acetonitrile (HPLC quality).
4. 10 m*M* dithiothreitol (DTT). A 1 *M* DTT solution is stable for month at -20 °C. Store as 25 µL aliquots and dilute 1:100 with 100 mM ammonium bicarbonate for use.
5. 55 mM iodoacetamide (Sigma,) in 100 mM ammonium bicarbonate. Has to be prepared freshly (iodoacetamide: 10.2 µg / µL).
6. 0.5 mL polypropylene tubes.
7. Zip-tips (Waters) or homemade micro-purification tips based on Poros 50 R2 reversed phase material (Applied BioSystems, #1-1159-05) and Eppendorf GELoader tips (Eppendorf, #0030 001.222) (*see* **Note 3**).

8. Vacuum centrifuge.
9. Concentrated solution of alpha-cyano-4-hydroxycinnamic acid (Aldrich) in 70% acetonitrile, 0.1% TFA in water.
10. MALDI mass spectrometer.

2.2. Alkylation of Protein

1. 8 *M* urea, 100 m*M* ammonium bicarbonate (or Tris-HCl), pH 8.
2. Reduction agent: typically dithiothreitol (DTT) is used for reduction, but mercaptoethanol or tris(carboxyethyl)phosphine (TCEP – Sigma) may be used instead. Mercaptoethanol is volatile while TCEP is also active at low pH.
3. Alkylating reagent: iodoacetate, iodoacetamide, 4-vinylpyridine and N-isopropyliodoacetamide are all commonly used reagents. 4-vinylpyridine has the disadvantage of being prone to polymerization and thus has a limited shelf life.

2.3. Peptide Mapping with Purification of Peptides

1) Suitable enzyme or chemistry (*see* **Table 1**).
2) Buffer for digestion (*see* **Note 4**)
3) HPLC system capable of binary gradient formation (*see* **Note 5**) equipped with an in-line UV detector and a strip-chart recorder and/or computer integrator.
4) Acetonitrile (HPLC grade, UV cutoff < 210 nm). Neat trifluoroacetic acid (TFA) (analytical grade is acceptable, sequencing grade is optimal) (*see* **Note 6**).
5) Automatic or manual fraction collection into polypropylene tubes.
6) Vacuum centrifuge

As many of the solvents and reagents are used in very small quantities (e.g. ≤ 1 mL) it can be advantageous to prepare stock solutions that are stored at –20 °C, even of those that are not specifically mentioned in the text.

Most proteolytic enzymes are supplied freeze dried. When first opened you may dissolve the enzyme in pure water, make 0.5-2 µg aliquots in small polypropylene tubes, and lyophilize the aliquots. Endo Glu-C protease may be stored frozen in pure water.

3. Methods

3.1. Selection of Proteolytic Enzyme

The selection of an enzyme for digestion is one of the most important considerations when performing peptide mapping, and should be carefully considered in relation to the task you want to perform.

For peptide mass fingerprinting (PMF) the most common choice is trypsin. This is one of the most active enzymes, it is well characterized for PMF analysis (*see* **Notes 8** and **15**), and cleaves with high specificity (C-terminal to Lys and Arg). Most tryptic peptides will have molecular masses in the range 800–2500 Da, which is well suited for mass spectrometry, Furthermore, you are certain that

Table 1
Proteolytic Enzymes Suitable for Peptide Mapping.

Name	EC no.	Size Da.	Type	Organism	Accession number	pH optimum	pH range	Requirements	Tolerates detergents	Cleavage	Specificity	Manufacturer	Note
Trypsin	3.4.21.4	23,293	Serine	Bovine	P00760	8	5.5 – 10.5	Stabilized by small amounts of Ca^{++}	2 M urea >0.1% SDS 50% acetonitrile	Arg, Lys	High	R,W,S	16
Trypsin	3.4.21.4	23,463	Serine	Porcine modified	P00761	8	5.5 – 10.5	Stabilized by small amounts of Ca^{++}	2 M urea >0.1% SDS 50% acetonitrile	Arg, Lys	High	A,S R, P,W, S, PS	16
Chymotrypsin A B	3.4.21.1	25,600 25,700	Serine	Bovine	P00766 P00767	8	7–9		0.1% SDS 1 M urea 10–30% acetonitrile	Phe, Trp, Tyr	Medium to high	R,A, W,S, PS	17
Endo Glu-C	3.4.21.9	27,000	Serine	Staphylococcus aureus V8	P04188	7.8	4–9		2 M urea 0.2% SDS 10% acetonitrile	Glu (Asp) -Glu	High	R,A,P, W,S, PS	18
Endo Lys-C	3.4.99.30	30,000	Serine	Lysobacter enzymogenes	P15636	8.5–8.8	7–9		0.1% SDS 1 M urea	Lys	Medium to high	R,S,PS	19

(continued)

Table 1 (continued)

Name	EC no.	Size Da.	Type	Organism	Accession number	pH optimum	pH range	Requirements	Tolerates detergents	Cleavage	Specificity	Manufacturer	Note
Endo Lys-N	3.4.24	22,000	Metallo	Grifola frondesa		10	6–10.5	Zn^{++} Avoid EDTA		N-terminal to Lys	High	SE	19
Lysyl endopeptidase	3.4.21.50	46.000	Serine	Achromabacter lyticus	P15636	8		Inhibited by ammonium salts	>0.1% SDS 4 M urea 40% acetonitrile	Lys	High	A	19
Endo Arg-C	3.4.21.40	30,000	Serine	Mouse submaxillary		8	7.5–8.5		2 M urea 10% acetonitrile	Arg	High	T,S,PS	19
Endo Asp-N	3.4.24.33	27.000	Metallo	Pseudomonas fragi		8	6.0–8.5	Avoid EDTA	0.1% SDS 1 M urea 10% acetonitrile	N-terminal to Asp	High	T,R,A,S, PS	19
Endo Asn-C		23.000	-SH	Jack Bean		5.0	4.5–6.5	1–10 mM dithiothreitol		Asn	High	T	19
Clostripain	3.4.22.8	50,000	-SH	Clostridium histolyticum	P09870	7.6		1–10 mM dithiothreitol	4 M urea 10% acetonitrile	Arg (Lys)	Medium	R,W,S	19

Pepsin	3.4.21.7	34,500	Acid	Pig gastric mucosa	P00791	2	1–5			N-terminal to hydrophobic Preferentially if preceded by Phe, Met, Leu, Trp	Low	R,A, W,S, PS	15
Thermolysin	3.4.24.4	37,500	Met-allo	Bacillus thermoproteolyticus	P00800	8	7–9	Ca^{++} Avoid EDTA	8 M urea	N-terminal to hydrophobic Not if followed by Pro	Low	A,S	15
Elastase	3.4.21.36	25.900	Serine	Pig pancreas	P00772	8.5	7–9		0.1% SDS 2 M urea	Uncharged (small) aliphatic	Low	R,A, W,S	15

Chemical cleavage procedures are not included in the table as experience shows that they tend to be rather unspecific for general peptide mapping, however, for those special hard to cleave proteins see relevant chapters in this book.

Accession number: Numbers refer to the Swiss-Prot/Uniprot database (http://www.ebi.ac.uk/).

Cleavage: All enzymes cleave C-terminal to the indicated residue except for except where noted. Minor, but still significant, secondary cleavages are listed in parenthesis. Cleavage does not take place if the cleavage position is followed by proline or a residue indicated by “-” (e.g. a -Lys-Pro- sequence is not cleaved by trypsin). See also notes for individual enzymes.

Specificity: Please *see* **Note 15** and notes for the individual enzymes.

Detergents: Even very small amounts of SDS is detrimental to mass spectrometric analysis, while reversed phase HPLC is capable of handling small amounts of SDS (*see* **Note 4**). When using urea you may dissolve the protein in a small amount of 8 M urea you can dilute to the required strength with buffer containing the relevant enzyme.

Manufacturer: R) Roche Biomolecular, S) Sigma Inc., P) Promega, T) Takara, W) Worthington, A) Wako, C) Calbiochem, PS) Princeton Separations all peptides will contain a basic residue (except the C-terminal peptide), which means they will ionize better when analyzed by mass spectrometry in positive polarity mode. Endo-Lys and in particular lysyl endopeptidase (higher activity and specificity) are also well suited for PMF, but the peptides generated will, on average, be much larger, which may impede their identification. However, in many cases you will increase the sequence coverage, particularly in basic regions of the protein. Other potential enzymes are Endo Asp-N and Endo Glu-C, but the results are usually much more unpredictable.

If the protein sequence of your target protein is known, you should look at the distribution of residues when aiming for the largest sequence coverage before selecting an enzyme. Particularly the charged residues are important, as most of the specific enzymes will target these residues (**Table 1**). A few computer programs for this type of analysis are available (*see* **Note 1**).

Important aspects for enzyme selection are:

- *Short peptides*. Very short peptides are difficult to handle, both by MALDI mass spectrometry (< 7 residues) and HPLC (< 5 residues if they are hydrophilic).
- *Long peptides*. The mass resolution and sensitivity decreases on the mass spectrometer as you get above 30–35 residues. Likewise large, hydrophobic peptides can be difficult to purify by reversed phase HPLC (> 50 residues). If your prime objective is to obtain amino acid sequence by Edman degradation, long peptides are generally most informative.
- Hydrophobic peptides tend to stick to the wall of polypropylene tubes, particularly if the sample is dried completely (*see* **Note 7**).
- If you want to do protein comparison, you should note that small peptides tend to emphasize differences (e.g. making it easier to identify small differences between proteins) while large peptides will emphasize similarity (e.g. make it easier to identify similar regions).
- Enzyme specificity, activity and buffer compatibility (see table 1) should be chosen relative to the intended analysis method (mass spectrometry, HPLC separation, Edman degradation, amino acid analysis etc.).
- For most proteins you will need at least two different enzymes (digests) in order to get a good coverage (> 90%).

3.2. Peptide Mass Mapping

1. Cut out the stained gel band or gel spot using a sharp scalpel or a properly sized puncher (2–4 mm diameter). Cut the spot into smaller pieces (1 mm square) and place them in a small polypropylene tube. Try to cut as close as possible to the actual spot. A small concentrated spot yields better results than a large diffuse one. If you have a one-dimensional gel band, you may not need the whole band, but can reserve part of it for additional experiments.
2. Wash the gel pieces carefully in 50 µL, 50 m*M* ammonium bicarbonate. Discard the supernatant.

3. Add 50 µL neat acetonitrile. Let stand for 5 min until the gel pieces has fully shrunken and become white. Withdraw the acetonitrile and discard. Dry the gel pieces in a vacuum centrifuge for 20 min.
4. Add 50 µL 10 m*M* dithiothreitol and incubate for 30 min at 56 °C.
5. Cool to room temperature and remove excess solvent.
6. Add 55 m*M* iodoacetamide in 100 m*M* ammonium bicarbonate so the gel pieces are just covered.
7. Let stand for 30 min in the dark before discarding the supernatant.
8. Wash the gel pieces in 20 µL 50% acetonitrile in water.
9. Add 30 µL neat acetonitrile. Mix until the gel pieces are fully shrunken and white. Remove the acetonitrile.
10. Add 20 µL 100 m*M* ammonium bicarbonate and mix for 5 min.
11. Add additional 30 µL neat acetonitrile and mix until the gel pieces are shrunken and white.
12. Remove the supernatant and dry the gel pieces in a vacuum centrifuge.
13. Add 12.5 ng/µL modified trypsin until the gel pieces are just covered with solvent. If you are using a low sensitivity stain like coomassie blue, the trypsin concentration should be doubled (*see* **Note 8**). Let stand for 5 min before removing excess trypsin containing solvent.
14. Add 50 m*M* ammonium bicarbonate until the gel pieces are just covered.
15. Let the digestion proceed overnight at 37 °C.
16. Extract the excess solvent, which will contain the peptides. Purify the peptides on a Zip tip (Waters) or for higher sensitivity use a home-packed GelLoader tip ***(3)*** using the recommended procedures (*see* **Note 3**).
17. Run the peptide map on a MALDI mass spectrometer in alpha cyano hydroxy cinnamic acid.
18. Analysis of the resulting mass spectra can easily be carried out using World Wide Web based tools (*see* **Note 9**), but be careful to check the results (*see* **Note 8**).

3.3. Reduction and Carboxymethylation of Proteins in Solution

1. Dried sample (20–100 µg) is dissolved in 20 µL 8 *M* urea, 0.4 *M* ammonium bicarbonate buffer (*see* **Note 10**).
2. Add 5 µl 45 m*M* dithiothreitol.
3. Stand at 50 °C for 15 min. Cool to room temperature.
4. Add 5 µL 100 m*M* iodoacetamide.
5. Stand at room temperature for 15 min in the dark.

Purify the protein by reversed phase HPLC (a short Poros R1 column, PerSeptive Biosystems, is well suited), gel filtration or reversed phase solid phase extraction.

For digestion with trypsin, endo Glu-C or lysyl endoproteinase (**Table 1**) you can proceed as follows without prior purification:

1. Add 140 µL water to dilute the urea to less than 2 *M*.
2. Add 2–4 % w/w of the selected enzyme. For prolonged digestion you can add the enzyme in two equal portions with an interval of 4–6 h.

3. Stand for 16–24 h. at 37 °C.
4. Freeze the sample or inject directly onto reversed phase HPLC column. When programming the separation gradient you should start with 2–5 min of isocratic elution in order to let the urea elute from the column before starting the separation of the peptides.

3.4. Digesting in Solution

If your protein contains disulfide bridges you should reduce and alkylate the protein prior to digestion, see above.

1. Dissolve the protein in an appropriate buffer (50 m*M* ammonium bicarbonate, *see* **Note 4**) to a concentration of 1–5 µg/µL. If you have less than 20 µg, you should dissolve in 20 µL.
2. Add the enzyme of your choice **(Table 1)** to a final concentration of 2–4% w/w. Hard to digest proteins and dilute solutions will need a higher enzyme to substrate ratio. For limiting the cleavage to a single or a few positions you can try to lower the concentration to 1% or lower.
3. Most proteins will be digested in 6–8 hours at 37 °C, but conditions may have to be varied to suit each individual protein. For hard to digest proteins you can use extended digestion times (e.g. 24–48 hours or longer) (*see* **Note 4**). For long digestion times it is advantageous to add the enzyme in two equal portions separated by 4–8 hours.
4. Digestions may be stopped by lowering the pH with the addition a small amount of 2% TFA (raising the pH to above 5 in the case of pepsin), by injecting the sample onto the HPLC system or by adding an appropriate enzyme inhibitor.
5. Separate the peptide map by reversed phase HPLC. Prior to separation you may want to extract ½ µL of your sample for MALDI analysis in order to check the extent of proteolysis.

A control reaction run under identical conditions as the main digest should be run in parallel to show which peptides comes from the sample and which are autodigest products or other contaminants. A control digest of a known protein (e.g. beta-lactoglobulin, **Fig. 1**) can also be run to check the activity of the protease.

A special case of peptide mapping is when you want to determine the disulfide bridge structure of a protein.

1. Verify the complete protein structure using standard peptide mapping techniques
2. Use a software tool to determine the optimal cleavage pattern of the target protein (*see* **Note 1**). Remember that disulfide interchange is minimized at low pH. Enzymes like trypsin, Endo Glu-C, Endo Asp-N, Endo Asn-C and pepsin are all active below a pH of 7 and are thus to be preferred.
3. Digest your protein as described above and separate half the sample by reversed phase HPLC.
4. Make the other half of the sample 10 m*M* in dithiothreitol or TCEP (will reduce you sample even at low pH). Let stand for at least 30 min before separation the sample under conditions identical to the separation in step 3).

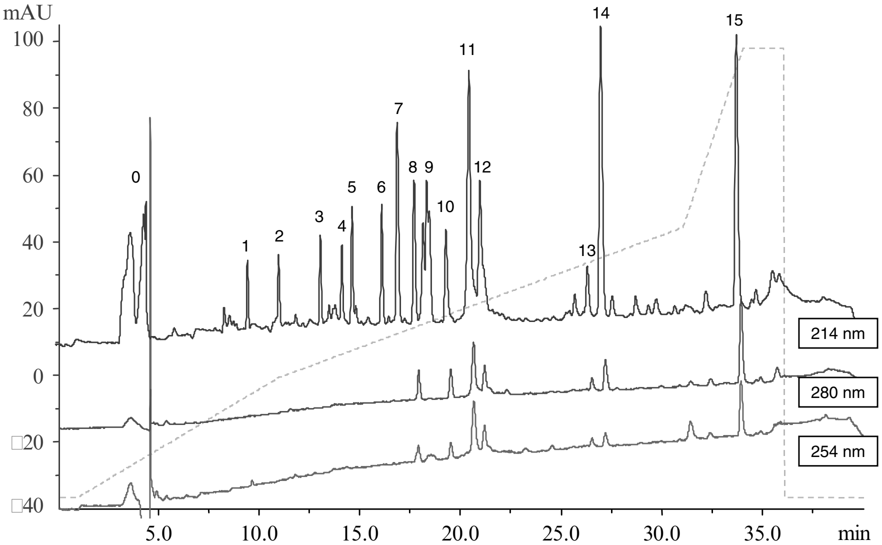

Fig. 1. Tryptic digest of 250 pmol bovine beta lactoglobulin. The traces of 214 nm, 254 nm and 280 nm are shown. The 254 nm and 280 nm traces are expanded x5 compared to the 214 nm trace. The stippled line show the gradient used. The identification of each peak is shown in figure 2. Peak 1 and peak 13 were not identified. Peak 13 is likely to be a partial digest product. The peaks at '0' are a mixture of small peptides and the buffer salts from the injection.

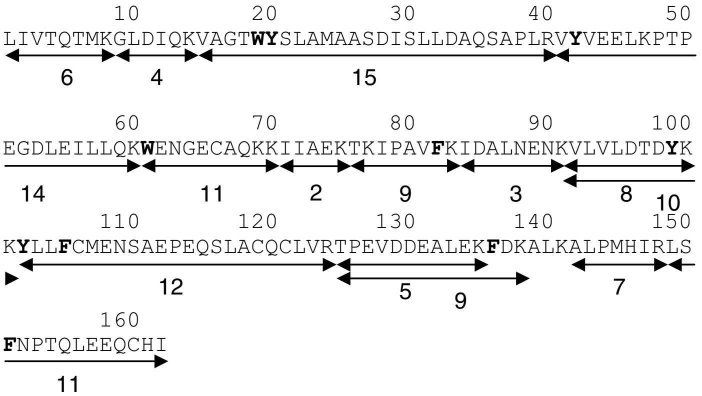
10 20 30 40 50
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTP
6
4
15
60 70 80 90 100
EGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYK
14
11
2
9
3
8
10
110 120 130 140 150
KYLLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLS
12
5
9
7
160
FNPTQLEEQCHI
11

Fig. 2. Identification of the peaks separated in figure 1. Aromatic residues are shown in bold. The two peptides labeled 11 are linked by a disulphide bond (Cys66–Cys160). Peptide 12 has an internal disulphide bond. The peptides labeled 9 elute very close together (fig. 1).

5. Compare the two chromatograms. Peaks that disappear from the first chromatogram and appear in the second chromatogram must arise from Cys containing peptides and will thus have to be identified by mass spectrometry, Edman degradation or amino acid analysis (*see* **Note 10**).
6. Closely spaced Cys residues in the primary structure presents a special problem as you will have to cleave between them for a positive identification of the disulfide bridge. In this case you will often have to isolate a large peptide containing multiple disulfide bridges followed by subdigestion using a less specific enzyme **(Table 1)** *(5)*.

Mass spectrometry is particularly suited to disulfide bridge determination and several methods have been developed *(7)*.

3.5. Peptide Map Purification by HPLC

Other chapters in this book deals in detail with the purification of proteins and peptides by HPLC, so the following only emphasis the features relevant to peptide mapping. When purifying a peptide map by HPLC, the objective is usually to obtain the highest possible resolution, sensitivity and yield, but not to handle large amounts. The following description should be read in this context.

For general peptide mapping use a large pore (300 Å), reversed phase column packed with the smallest possible particle size and the longest column length. These parameters are conflicting as backpressure in the system rises with both smaller particle size and longer column length. A compromise is to use a long 250 mm column packed with 5 µm particles or a shorter 150 mm column packed with 3 µm particles. Backpressure in the running system should not exceed 100 bar (increasing the temperature to 45-50 °C using a column oven will lower the back pressure and will additionally increase the resolution slightly).

Using smaller diameter columns will increase the sensitivity of separation, if the HPLC system is optimized for the lower flow, at the cost of lower capacity (*see* **Note 5**). Furthermore, the peak volume from a 2.1 mm column is about 100 µL compared to 400-500 µL from a 4.6 mm column. This makes the volumes more compatible for further downstream processing (e.g. mass spectrometry or Edman degradation) and speeds up lyophilization (*see* **Note 7**).

An in-line UV detector is essential for separating peptide mixtures. As we cannot be certain that all peptides contain aromatic residues, detection has to be carried out at a wavelength low enough to observe the absorbance of the peptide bond. However, as solvents like 0.1% TFA and acetonitrile also absorbs at low wavelength we need to find the wavelength with the highest signal to noise ratio. On most systems this is at 214-215 nm. If you do not operate with the highest purity of solvents, you may have to increase the wavelength to 218-220 nm, but your sensitivity will be reduced by a factor of three.

Multiple wavelength detection (e.g. 254 nm and 280 nm) can greatly increase the confidence of peptide identification (*see* **Note 11**).

3.5.1. Separating a Peptide Map

1. Prepare 1 liter of A-solvent (5% acetonitrile/0.1% TFA in ultra high quality water) and ½ liter of B-solvent (90% acetonitrile in water, 0.85% TFA) (*see* **Notes 6** and **12**). Degas solvents carefully before use or, better, use in-line degassing, as degassed solvents will re-absorb oxygen from the air within a few hours.
2. Program your HPLC system to a gradient of 1%/min from 0% to 40% B-solvent, followed by 2%/min from 40 to 70% B-solvent. Finally step to 100% B-solvent for 2 min before equilibrating at 5% for at least 10 column volumes until you have a stable baseline. If your peptide map is not too complex, you may shorten the run time by increasing the gradient to 1½%/min between 0 and 40% and 2.5–3%/min between 40 and 70%.
3. Make a blind run each morning to equilibrate the column. The first run of a day is usually atypical with a rising or falling baseline that may not be present in subsequent analyses. You can most easily check the condition of your HPLC system and reversed phase column by making a separation run using a standard peptide mixture (e.g. a tryptic digest of β-lactoglobulin) (*see* **Note 13**).
4. Dissolve your sample in A-solvent and inject. Keep the injector in the "inject" position for two injection loop volumes before you switch back to "load" position. If your sample is already dissolved in a different solvent (i.e. urea, tris or phosphate) you should allow 2–3 min of isocratic elution before starting the gradient.
5. Collect the isolated peaks in polypropylene tubes. If you run at low flow rates, you may have to take a delay between the detector and the effluent from the capillary into account (*see* **Note 14**).
6. Dry down your collected fractions in a vacuum centrifuge (*see* **Note 7**). For several applications you may not have to dry the sample; just evaporate the major part of the organic modifier.

For problems in peptide map separations, *see* **Note 2.**

4. Notes

1. When the primary structure of the protein being analyzed is known, a couple of software tools are available for planning the peptide map. These tools can predict the peptides generated by various proteolytic enzymes or chemical cleavages. One freeware tool, PAWS, is available for download (http://bioinformatics.genomicsolutions.com/Paws.html), while a somewhat more advanced tool, GPMAW (http://www.gpmaw.com) *(7)*, will allow you to work with multiple sequences and, in addition to the prediction of peptides, provides additional physical/chemical characteristics of each peptide (e.g. charge, theoretical pI, hydrophobicity and size). GPMAW can even generate a simulated HPLC RP chromatogram. Although this chromatogram is only approximate (separation varies with column, system and

running conditions), it does give valuable indications for problems during separation.

The Expasy web site (http://www.expasy.ch/tools/) contains links to a large number of tools that are useful for peptide mapping (most are included in GPMAW).

2. If your peptide map contains small hydrophilic peptides they will elute very early in the reversed phase chromatogram, perhaps even as part of the solvent peak. Early eluting peaks may also have a bad peak shape, particularly if the column has not been equilibrated sufficiently before the start of the gradient. You may collect these early eluting peaks, dry them sufficiently to get rid of the organic solvent, and then re-separate on a 100–120Å pore size, high carbon load column (e.g. Hypersil ODS2 or equivalent) using 0.1%TFA / 90% methanol as a solvent system. The gradient should be a rather shallow 1%/min. gradient from 0 to 30%. An alternative is to separate hydrophilic peptides using HILIC (hydrophilic interaction chromatography), particularly glycopeptides can be efficiently separated in this way. Running HILIC chromatography is very similar to reversed phase chromatography, but the elution order of components is reversed. You dissolve your sample in high concentration of organic solvent and separate by running the gradient from high to low concentration of organic solvent.

   If on the other hand you have large, hydrophobic peptides you should use a 300Å, C4 or C8 column and elute with either acetonitrile or, for the really hydrophobic peptides, use 2-propanol.

3. The use of reversed phase purification as the last step before analysis of peptide mixtures is necessary to obtain the highest sensitivity and sequence coverage in mass spectrometric peptide mapping. You may use Zip-tips (Waters Inc.), but when analyzing peptide maps isolated from silver stained gel spots you can obtain considerable higher sensitivity by using homemade micro purification tips based on Eppendorf GELoader tips packed with Poros R2 50 µm reversed phase material (Applied BioSystems, Framingham, MA). For the purification of proteins you should use Poros R1 material. For details on how to construct these devices please see ***(8)***

   The GELoader tip is constricted at the end using the blunt end of a pair of tweezers or a pencil. Column material is dispersed in acetonitrile and sufficient material is applied to make a 3–5 mm length column. The GELoader tip is initially washed with 10–20 µL of neat acetonitrile, and then it is equilibrated in 2 × 10 µL of 0.1% TFA. The sample is added in 1–20 µL of solvent followed by washing with 2 × 10 µL of 0.1% TFA. Finally the sample is eluted with 0.8–1 µL of alpha-cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.1% TFA (4 mg/mL to concentrated depending on instrument) directly onto the MALDI target.

4. Most buffers without an excessive amount of detergent or salt are suitable for proteolytic digestion. 50 m*M* ammonium bicarbonate is convenient because it can be removed by evaporation (do not use with lysyl endopeptidase). If you have large quantities or high concentrations of ammonium bicarbonate you may need to add a bit of acetic acid to help completely remove the ammonium bicarbonate. This buffer may be readily substituted for other buffers, e.g. 50 m*M* Tris-HCl, pH 8, or 50 m*M* phosphate buffer, pH 8.0. However, these buffers are not directly compatible with mass spectrometry.

   Hard to dissolve proteins can often be brought into solution by first dissolving the protein in small amount of 6–8 *M* urea (e.g. 5–10 μL) and then diluting the solution to the appropriate level (e.g. below 2 *M* for trypsin, *see* **Table 1**) with a buffer containing the enzyme for digestion. Another very efficient method is to use detergents, however, beware of downstream processing incompatibilities (e.g. SDS is incompatible with mass spectrometry and HPLC). RapiGest SF (Waters) and PPS Silent™ (Protein Discovery) are detergents that upon acidification degrade into smaller components which makes them compatible with both mass spectrometry and HPLC. Organic solvents (*see* **Table 1**) or heat denaturation may denature proteins, thus leading to faster, more efficient digestions ***(9)***.
5. The sensitivity of HPLC separations decreases with the square of the column diameter, thus a 2 mm column shows four times the sensitivity of a 4 mm column, if the HPLC system is built to handle the smaller diameter. As the column diameter decreases from 4 mm to 2 and 1 mm the optimal flow rate decreases from 1 mL/min to 0.25 mL/min and 0.06 mL/min. Unless you perform flow splitting you will need special HPLC pumps to be able to perform a gradient in 0.06 mL/min. In addition, the rest of the HPLC equipment has to be optimized for the lower flow rate in order to minimize extra column band broadening. The inner diameter of capillaries has to be decreased (*see* **Table 2**). You also need to make connections as short as possible, particularly after

Table 2
Parameters for HPLC System for Binary Gradient Formation

	Column diameter	Typical flow mL/min	Flow cell volume	Capillary diameter	Sensitivity increase
Normal	4–4.6 mm	1.0	< 12 μL	0.25 mm / 0.10”	× 1
Narrow	2–2.1 mm	0.2–0.25	< 8 μL	0.18 mm / 0.07”	× 4
Micro	1 mm	0.05–0.07	< 2 μL	0.12 mm / 0.05”	× 16

the column. The size of the detector flow cell is particularly important; as a standard flow cell with a volume of 10–12 µL will significantly decrease the resolution of a 2 mm column, even though the rest of the system is optimized (*see* **Table 2**). When changing to a smaller diameter column you may also have to replace the dynamic mixer as you may otherwise experience very long delay times.

For dual pump systems, each pump should have a minimum flow of no more than 5% of the running flow (e.g. a minimum flow of no more than 12.5 µL/min for a set total flow of 250 µL/min), and a minimum step of no more than 1% of the set flow rate. You may manage with slightly lower performance as the dynamic mixer will smooth out irregularities, but particularly at the beginning of the gradient you may observe irregular separation behavior.

6. TFA deteriorates over time. As the 0.1 % TFA used for buffer A ages you will see an increase in baseline during gradient runs. You may increase/decrease the content of TFA in the B-solvent to compensate. A ratio of 1:0.85 is usually a good starting point, but varies slightly from detector to detector. If you experience regular baseline noise, you can try to lower the content of TFA in buffer A to 0.06% and the content in buffer B correspondingly (0.05%).
7. For small amounts of peptide, the drying step can be critical for recovery as the peptides have a tendency to adhere to the walls of the polypropylene tube ***(10)***. For many analytical purposes it is not necessary to change solvent or you may only have to evaporate the organic solvent.
8. An accurate calibration of your mass spectra is essential for correct protein identification. The most accurate calibration is to use known peaks in each spectrum for internal calibration. When using Promega modified trypsin (porcine) you will find the following autolytic peptides (monoisotopic m/z) that are suited for internal calibration: 842.510, 1045.564 and 2211.105. If these peaks in general are too high or too low, you should adjust the concentration (not the volume) of trypsin added in step 13.

   When analyzing the results of a search, you should consider the following when differentiating between hits:

   How do the deviations between theoretical and experimental masses fit together (i.e. do the deviations lie on a straight line when plotted against the peptide mass you have a calibration error).

   If a peptide contains a methionine it is likely to have a +16 Da peak. The oxidized methionine seldomly occurs by itself.

   N-terminal glutamine containing peptides are always accompanied by a −17 Da satellite peak (deamidation to pyroglutamic acid).

   Multibasic sites often give rise to lysine/arginine ladders (tryptic cleavage).

Missed cleavages (i.e. peptides with an internal Lys or Arg residue) are much more likely to occur if there is a neighboring acidic residue (tryptic cleavage).

In a peptide mixture, (small) lysine-containing peptides are often suppressed relative to arginine containing peptides due to the more basic nature of arginine.

The GPMAW program (*see* **Note 1**) is also useful for checking the validity of a hit as you can perform detailed analysis of likely/unlikely peptide identifications as well as search for unusual enzyme cleavage sites.

9. Several Web sites cater for the analysis of peptide mass data. Some analyze MS data (PMF) others MS/MS data. The actual number changes over time, but you may want to check out the following:

   Mascot (http://www.matrixscience.com)
   ProteinProspector (MsFit) (http://prospector.ucsf.edu/).
   Aldente (http://www.expasy.org/tools/aldente/)
   PeptideSearch (http://www.mann.embl-heidelberg.de/GroupPages/pageLink/peptidesearchpage.html)
   XTandem (http://www.thegpm.org/)
   VEMS (http://yass.sdu.dk/)
   Phenyx (http://phenyx.vital-it.ch/pwi/login/login.jsp)

   You will have to read the accompanying help files for each search engine for detailed instructions.

10. You cannot usually identify reduced cysteine residues by Edman degradation or amino acid analysis, but the surrounding sequence will in most cases be sufficiently unique that positive identification of the peptide is no problem.
11. When separating a peptide map, it is advantageous to use multiple wavelengths. Modern in-line UV detectors can often be set up for three simultaneous wavelengths. You should use 214 nm for separating peptides, as you will get the highest response at this wavelength (you are measuring the absorbance of the peptide bond). Only the aromatic amino acid residues have any significant absorption at higher wavelength. Tryptophan and tyrosine have absorption maxima at 278 nm and 280 nm respectively while phenylalanine has maximum absorbance at 254 nm. You should thus set your detector at 214 nm, 254 nm and 280 nm, Tryptophan is usually recognized by having an absorbance several times that of tyrosine.
12. The inclusion of TFA in the B-solvent is in order to create a stable baseline as both acetonitrile and TFA absorbs at low wavelength. Depending on your system you may have to vary the concentration of TFA in the B-solvent between 0.75 and 0.85 % (*see* **Note 6**).

    Mixing a small amount of organic solvent in the A-solvent and some water in the B-solvent helps to give a better mixing of the solvents (mixing water and acetonitrile is quite difficult), gives a better separation (helps to "wet" the hydrophobic surface, it may "collapse" in pure water) and helps to degas the A-solvent. If you have hydrophilic peptides that are eluted in the solvent front by the initial 5% organic content, you can use an A-solvent without organic content, but the first part of the chromatogram tend not to be so reproducible.

Column manufacturers recommend that you store the column in 50% methanol/water. However, experience has shown that most columns are stable for several years when stored in 0.1% TFA/acetonitrile (preferably with a high content of organic solvent).

13. Easy-to-make test mixture of peptides for checking reversed phase columns for peptide mapping: Dissolve 182 µg β-lactoglobulin (Sigma Inc, #L0130) in 100 µL, 50 m*M* ammoniumbicarbonate, pH 8.2. Add 3.6 µg trypsin (Roche Biochemicals, #109 819) and digest for 4 hours at 37 °C. Stop the digestion with 900 µL 0.1% TFA. Total solution is now 1000 µL at a concentration of 10 pmol /µL. Divide into 55 µL portions and store at −20 °C. Use 50 µL for 4 mm columns and 20 µL for 2 mm columns. The digest is also useful as calibrant/check for MALDI-MS.
14. If you use an automatic fraction collector you should be careful to calibrate the fraction collector for exact peak collection. In addition you should be careful that the collector adds a minimum of additional peak tubing to the separation system as this will result in delays and mixing of your sample resulting in a loss of resolution. Manual fraction collection is usually more precise but you may (initially) need an assistant to assist in tube handling and annotation. For manual collection a strip chart recorder is most convenient, as computer acquisition or integrators often have an unacceptable delay.
15. Most proteolytic enzymes do not have an absolute specificity, but are active toward several residues with varying activities. Activity towards a given type of residue is usually also dependent on the local environment (e.g. 3-D structure and neighboring residues). Excessive digestion times may thus lead to secondary cleavages that are not desired and may be difficult to predict.

    Most of the enzymes that have a single residue specificity (Lys, Arg, Glu, Asp, Asn) will show some level of activity towards "like" residues.

    While the selection of the specific enzymes can be clearly related to peptide predictions based on the primary structure (e.g. using the GPMAW program), the selection of unspecific enzymes like pepsin, thermolysin and elastase is usually based on other parameters like low solubility of the target protein. They have a difficult to predict, low specificity that is very dependent on the local structure of the substrate. Pepsin is active at pH 2.0, a pH where proteins often are more soluble than at neutral pH.

Thermolysin is active at temperatures as high as 80 °C (even in the presence of 8 *M* urea) where most proteins are denatured. Elastase targets a group of small hydrophilic residues not targeted by any of the "specific" enzymes. These enzymes are usually used either for an initial cleavage where you hope for few cleavage positions to either make a protein soluble or to cleave a large protein into smaller, more manageable pieces. In these cases you have to experiment with short digestion times and/or very dilute solutions. Alternatively the enzymes are used as a final cleavage to identify closely spaced disulfide bridges (see step 4).

16. During digestion, trypsin will autolyse and generate a small amount of ψ-trypsin, which has some chymotryptic activity. In certain cases, depending on the sequence or residual structure of the substrate, this may lead to complete cleavage at single or a few residues in a protein. The activity of trypsin is also affected by neighboring residues as acidic groups will slow cleavages and a following proline residue will in most cases completely inhibit cleavage. If you have neighboring basic residues (e.g. -Lys-Lys-) trypsin may cleave at either residue, but as the activity of trypsin towards terminal residues is considerably lower than towards internal residues, you may find all cleavage variants present in the peptide map even after prolonged digestion times.

    Porcine trypsin is usually preferred rather than bovine, due to less autolysis. Modified trypsin, which is even more resistant to autolysis, is available from a number of manufacturers (**Table 1**).
17. Chymotrypsin has a high activity towards the C-terminal side of aromatic residues, but other residues like Leu and Met can also be cleaved, particularly if they are situated in a hydrophobic environment (mostly -X-X↓Y where X is hydrophobic). Full digestions of intact proteins often give rise to unsuspected peptides, while the specificity when sub-digesting peptides is usually quite high.
18. Endoproteinase Glu-C, also called *S. aureus* protease V8, is a most useful enzyme in protein mapping. Its specificity nicely complements that of trypsin while still generating peptides of a suitable size. The main activity is directed towards the C-terminal of glutamic acid, but a reasonable activity towards aspartic acid means that you may have to control digestion times carefully in order to limit cleavage to Glu. In a few cases activity has also been seen towards Gly, but this is sequence dependent. If the following residue is Glu or Pro, cleavage will not take place (e.g. in a -Glu-Glu-sequence cleavage is only observed after the second Glu). Earlier reports that Endo Glu-C was specific towards Glu at pH 4.0 seem to be caused by the lower activity of the enzyme at this pH ***(11)***.
19. Lysyl endopeptidase, Endo Lys-C, Endo Asp-N, Endo Lys-N, and Endo Asn-C all tend to generate very clean peptide maps with a low amount of secondary cleavages. However, some of the peptides generated may be quite large

and difficult to handle. Endo Asp-N, Endo Lys-N, and Endo Asn-C are considerably more expensive than the other enzymes mentioned but a single optimal digest is more likely to succeed than multiple rounds of digestion and separation.

Endo Arg-C (submaxillary gland proteinase) and clostripain both target the C-terminal side of arginine, but with a significant amount of lysine cleavage. Furthermore, their activity is not very high making them problematic for digesting intact proteins.

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Production of Protein Hydrolysates Using Enzymes

John M. Walker and Patricia J. Sweeney

1. Introduction

Traditionally, protein hydrolysates for amino acid analysis are produced by hydrolysis in 6 *N* HCl. However, this method has the disadvantage that tryptophan is totally destroyed, serine and threonine partially (5–10%) destroyed, and most importantly, asparagine and glutamine are hydrolyzed to the corresponding acids. Digestion of the protein/peptide with enzymes to produce protein hydrolysate overcomes these problems, and is particularly useful when the concentration of asparagine and glutamine is required. For peptides less than about 35 residues in size, complete digestion can be achieved by digestion with aminopeptidase M and prolidase. For larger polypeptides and proteins, an initial digestion with the non-specific protease Pronase is required, followed by treatment with aminopeptidase M and prolidase. Since it is important that all enzymes have maximum activity, the following sections will discuss the general characteristics of these enzymes.

1.1. Pronase

Pronase (EC 3.4.24.4) is the name given to a group of proteolytic enzymes that are produced in the culture supernatant of *Streptomyces griseus* K-1 ***(1–3)***. Pronase is known to contain at least ten proteolytic components: five serine-type proteases, two Zn^{2+} endopeptidases, two Zn^{2+}-leucine aminopeptidases, and one Zn^{2+} carboxypeptidase ***(4, 5)***. Pronase therefore has very broad specificity, hence its use in cases where extensive or complete degradation of protein is required. The enzyme has optimal activity at pH 7.0–8.0. However, individual components are reported to retain activity over a much wider pH range ***(6–9)***. The neutral components are stable in the pH range 5.0–9.0 in the presence of calcium, and have optimal activity at pH 7.0–8.0. The alkaline components are stable in the pH range

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3.0–9.0 in the presence of calcium, and have optimal activity at pH 9.0–10.0 ***(4)***. The aminopeptidase and carboxypeptidase components are stable at pH 5.0–8.0 in the presence of calcium ***(9)***. Calcium ion dependence for the stability of some of the components (mainly exopeptidases) was one of the earliest observations made of Pronase ***(2)***. Pronase is therefore normally used in the presence of 5–20 m*M* calcium. The addition of excess EDTA results in the irreversible loss of 70% of proteolytic activity ***(10)***. Two peptidase components are inactivated by EDTA, but activity is restored by the addition of Co^{2+} or Ca^{2+}. One of these components, the leucine aminopeptidase, is heat stable up to 70°C. All other components of Pronase lose 90% of their activity at this temperature ***(5)***. The leucine aminopeptidase is not inactivated by 9 *M* urea, but is labile on dialysis against distilled water ***(2)***. Some of the other components of Pronase are also reported to be stable in 8 *M* urea ***(2)***, and one of the serine proteases retains activity in 6 *M* guanidinium chloride ***(11)***. Pronase retains activity in 1% SDS (w/v) and 1% Triton (w/v) ***(12)***.

Among the alkaline proteases, there are at least three that are inhibited by diisopropyl phosphofluoridate (DFP) ***(10)***. In general, the neutral proteinases are inhibited by EDTA, and the alkaline proteinases are inhibited by DFP ***(4)***. No single enzyme inhibitor will inhibit all the proteolytic activity in a Pronase sample.

1.2. Aminopeptidase M

Aminopeptidase M (EC 3.4.11.2), a zinc-containing metalloprotease, from swine kidney microsomes ***(13–16)*** removes amino acids sequentially from the N-terminus of peptides and proteins. The enzyme cleaves N-terminal residues from all peptides having a free α-amino or α-imino group. However, in peptides containing an X-Pro sequence, where X is a bulky hydrophobic residue (Leu, Tyr, Trp, Met sulfone), or in the case of an N-blocked amino acid, cleavage does not occur. It is for this reason that prolidase is used in conjunction with aminopeptidase M to produce total hydrolysis of peptides. The enzyme is stable at pH 7.0 at temperatures up to 65°C, and is stable between pH 3.5 and 11.0 at room temperature for at least 3 h ***(15)***. It is not affected by sulfhydryl reagents, has no requirements for divalent metal ions, is stable in the presence of trypsin, and is active in 6 *M* urea. It is not inhibited by PMSF, DFP, or PCMB. It is, however, irreversibly denatured by alcohols and acetone, and 0.5 *M* guanidinium chloride, but cannot be precipitated by trichloroacetic acid ***(15)***. It is inhibited by 1,10-phenanthroline (10M) ***(16)***.

Alternative names for the enzyme are amino acid arylamidase, microsomal alanyl aminopeptidase, and α-aminoacyl peptide hydrolase.

1.3. Prolidase

Prolidase (EC 3.4.13.9) is highly specific, and cleaves dipeptides with a prolyl or hydroxyprolyl residue in the carboxyl-terminal position ***(17, 18)***. It has no activity with tripeptides ***(19)***. The rate of release is inversely proportional to the size of the amino-terminal residue ***(19)***. The enzyme's activity depends on the nature of the amino acids bound to the imino acid. For optimal activity, amino acid side chains must be as small as possible and apolar to avoid steric competition with the enzyme receptor site. The enzyme has the best affinity for alanyl proline and glycyl proline. The enzyme has optimal activity at pH 6.0–8.0, but it is normally used at pH 7.8–8.0 ***(20)***. Manganous ions are essential for optimal catalytic activity. The enzyme is inhibited by 4-chloromercuribenzoic acid, iodoacetamide, EDTA, fluoride, and citrate. However, if Mn^{2+} is added before iodoacetamide, no inhibition is observed ***(21)***.

Alternative names for the enzyme are imidodipeptidase, proline dipeptidase, amino acyl L-proline hydrolyase, and peptidase D.

2. Materials

1. Buffer: 0.05 *M* ammonium bicarbonate, pH 8.0 (no pH adjustment needed) or 0.2 *M* sodium phosphate, pH 7.0 (*see* **Note 1**).
2. Pronase: The enzyme is stable at 4°C for at least 6 mo and is usually stored as a stock solution of 5–20 mg/mL in water at −20°C.
3. Aminopeptidase M: The lyophilized enzyme is stable for several years at −20°C. A working solution can be prepared by dissolving about 0.25 mg of protein in 1 mL of deionized water to give a solution of approx 6 U of activity/mL. This solution can be aliquoted and stored frozen for several months at −20°C.
4. Prolidase: The lyophilized enzyme is stable for many months when stored at −20°C and is stable for several weeks at 4°C if stored in the presence of 2 m*M* $MnCl_2$ and 2 m*M* β-mercaptoethanol ***(18)***.

3. Methods

3.1. Digestion of Proteins (22)

1. Dissolve 0.2-µmol of protein in 0.2 mL of 0.05*M* ammonium bicarbonate buffer, pH 8.0, or 0.2 *M* sodium phosphate, pH 7.0 (*see* **Note 1**).
2. Add Pronase to 1% (w/w), and incubate at 37°C for 24 h.
3. Add aminopeptidase M at 4% (w/w), and incubate at 37°C for a further 18 h.
4. Since in many cases the X-Pro- bond is not completely cleaved by these enzymes, to ensure complete cleavage of proline-containing polypeptides, the aminopeptidase M digest should be finally treated with 1 µg of prolidase for 2 h at 37°C.
5. The sample can now be lyophilized and is ready for amino acid analysis (*see* **Note 2**).

3.2. Digestion of Peptides (22)

This procedure is appropriate for polypeptides less than about 35 residues in length. For larger polypeptides, use the procedure described in **Subheading 3.1.**

1. Dissolve the polypeptides (1 nmol) in 24 µL of 0.2 *M* sodium phosphate buffer, pH 7.0, or 0.05 *M* ammonium bicarbonate buffer, pH 8.0 (*see* **Note 1**).

2. Add 1 µg of aminopeptidase M (1 µL), and incubate at 37°C.
3. For peptides containing 2–10 residues, 8 h are sufficient for complete digestion. For larger peptides (11–35 residues), a further addition of enzyme after 8 h is needed, followed by a further 16-h incubation.
4. To ensure complete cleavage at proline residues, finally treat the digest with 1 µg of prolidase for 2 h at 37°C.
5. The sample can now be lyophilized and is ready for amino acid analysis (*see* **Note 2**).

4. Notes

1. Sodium phosphate buffer should be used if ammonia interferes with the amino acid analysis.
2. When using two enzymes or more, there is often an increase in the background amino acids owing to hydrolysis of each enzyme. It is therefore important to carry out a digestion blank to correct for these background amino acids.

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Amino Acid Analysis by Precolumn Derivatization with 1-Fluoro-2,4-Dinitrophenyl-5-L-Alanine Amide (Marfey's Reagent)

Sunil Kochhar and Philipp Christen

1. Introduction

Precolumn modification of amino acids and the subsequent resolution of their derivatives by reverse-phase high-performance liquid chromatography (RP-HPLC) is the preferred method for quantitative amino acid analysis. The derivatization step introduces covalently bound chromophores necessary not only for interactions with the apolar stationary phase for high resolution but also for photometric or fluorometric detection.

Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide or (*S*)-2-[(5-fluoro-2,4-dinitrophenyl)-amino]propanamide, is used to separate and to determine enantiomeric amino acids *(1)*. The reagent reacts stoichiometrically with the amino group of enantiomeric amino acids to produce stable diastereomeric derivatives, which can readily be separated by reverse-phase HPLC (**Fig. 1**). The dinitrophenyl alanine amide moiety strongly absorbs at 340 nm ($\varepsilon = 30{,}000\,M^{-1}\,cm^{-1}$), allowing detection in the subnanomolar range. With smaller columns, the sensitivity will correspondly increase.

Pre-column derivatization with the reagent can also be used to quantify the 19 commonly analyzed L-amino acids *(2)*. Major advantages of Marfey's reagent over other pre-column derivatizations are: (1) possibility to carry out chromatography on any multipurpose HPLC instrument without column heating; (2) photometric detection at 340 nm that is insensitive to most solvent impurities; and (3) stable amino acid derivatives. For the occasional user, the simple methodology provides, an attractive and inexpensive alternative to the dedicated amino acid analyzer. Further development to on-line derivatization and microbore chromatography has been reported *(3)*. The pre-column derivatization

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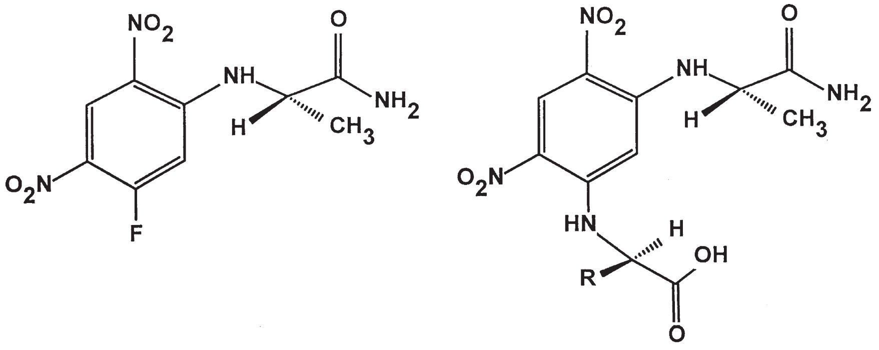

I II

Fig. 1. Marfey's reagent (I) and L,L-diastereomer derivative from L-amino acid and the reagent (II).

with Marfey's reagent has found applications in many diverse areas of biochemical research ***(4–16;*** for recent reviews, see ***17, 18***), including determination of substrates and products in enzymic reactions of amino acids (*see* **Note 1**).

2. Materials

2.1. Vapor-Phase Protein Hydrolysis

1. Pyrex glass vials (25–50 × 5 mm) from Corning.
2. Screw-capped glass vials.
3. 6*N* HCl 1.from Pierce.

2.2. Derivatization Reaction

1. Amino acid standard solution H from Pierce.
2. Marfey's reagent from Pierce. **Caution: Marfey's reagent is a derivative of 1- fluoro-2,4-dinitrobenzene, a suspected carcinogen.** Recommended precautions should be followed in its handling ***(19)***.
3. HPLC/spectroscopic grade triethylamine, methanol, acetone, and dimethyl sulfoxide (DMSO) from Fluka.

2.3. Chromatographic Analysis

1. Solvent delivery system: Any typical HPLC system available from a number of manufacturers can be used for resolution of derivatized amino acids. We have used an HPLC systems from Bio-Rad (HRLC 800 equipped with an autoinjector AST 100), Hewlett-Packard 1050 system and Waters system.
2. Column: A silica-based C_8 reverse-phase column, for example, LiChrospher 100 RP 8 (250 × 4.6 mm; 5 µm from Macherey-Nagel), Aquapore RP 300 (220 × 4.6 mm; 7 µm from Perkin-Elmer), Nucleosil 100-C_8 (250 × 4.6 mm; 5 µm from Macherey-Nagel), or Vydac C_8 (250 × 4.6 mm; 5 µm from The Sep/a/ra/tions group).
3. Detector: A UV/VIS HPLC detector equipped with a flow cell of lightpath 0.5–1 cm and a total volume of 3–10 µL (e.g., Bio-Rad 1790 UV/VIS monitor,

Hewlett-Packard Photodiode Array 1050, Waters Photodiode Array 996). The derivatized amino acids are detected at 340 nm.

4. Peak integration: Standard PC-based HPLC software with data analysis program can be employed to integrate and quantify the amino acid peaks (e.g., ValueChrom from Bio-Rad, Chemstation from Hewlett-Packard or Millennium from Waters).
5. Solvents: The solvents should be prepared with HPLC grade water and degassed.

a. Solvent A: 13 m*M* trifluoroacetic acid plus 4% (v/v) tetrahydrofuran in water.
b. Solvent B: Acetonitrile (50% v/v) in solvent A.

3. Methods

3.1. Vapor-Phase Protein Hydrolysis

1. Transfer 50–100 pmol of protein sample or 20 µL of amino acid standard solution H containing 2.5 µmol/mL each of 17 amino acids into glass vials and dry under reduced pressure in a SpeedVac concentrator.
2. Place the sample vials in a screw cap glass vial containing 200 µL of 6 *N* HCl.
3. Flush the vial with argon for 5–15 min and cap it airtight.
4. Incubate the glass vial at 110 °C for 24 h or 150 °C for 2 h in a dry-block heater.
5. After hydrolysis, remove the glass vials from the heater, cool to room temperature, and open them slowly.
6. Remove the insert vials, wipe their outside clean with a soft tissue paper, and dry under reduced pressure.

3.2. Derivatization Reaction

1. Add 50 µL of triethylamine/methanol/water (1:1:2) to the dried sample vials, mix vigorously by vortexing, and dry them under reduced pressure (*see* **Note 2**).
2. Prepare derivatization reagent solution (18.4 m*M*) by dissolving 5 mg of Marfey's reagent in 1 mL of acetone.
3. Dissolve the dried amino acid mixture or the hydrolyzed protein sample in 100 µL of 25% (v/v) triethylamine in water and add 100 µL of the Marfey's reagent solution and mix by vortexing (*see* **Note 3**).
4. Incubate the reaction vial at 40 °C for 60 min with gentle shaking protected from light (*see* **Note 4**).
5. After incubation, stop the reaction by adding 20 µL of 2 *N* HCl (*see* **Note 5**). Dry the reaction mixture under reduced pressure.
6. Store the dried samples at –20 °C in the dark until used (*see* **Note 6**).
7. Dissolve the dry sample in 50% (v/v) DMSO.

3.3. Chromatographic Analysis

1. Prime the HPLC system according to the manufacturer's instructions with solvent A and solvent B.
2. Equilibrate the column and detector with 90% solvent A and 10% solvent B.
3. Bring samples for analysis to room temperature; dilute, if necessary, with solvent A, and inject 20 µL (50–1000 pmol) onto the column (*see* **Note 7**).

4. Elute with gradient as described in **Table 1** (*see* **Note 8**). Resolution of 2,4-dinitrophenyl-5-L-alanine amide derivatives of 18 commonly occurring L-amino acids and of cysteic acid is achieved within 120 min (**Fig. 2**) (*see* **Note 9**).
5. Determine the response factor for each amino acid from the average peak area of standard amino acid chromatograms at 100, 250, 500, and 1000 pmol amounts.
6. When HPLC is completed, wash the column and fill the pumps with 20% (v/v) degassed methanol for storage of the system. Before re-use, purge with 100% methanol.

Table 1
HPLC Program for Resolution of Amino Acids Derivatized with Marfey's Reagent

Time (min)	% Solvent A	% Solvent B	Input
0	90	10	
15	90	10	Detector auto zero Inject
15.1	90	10	
115	50	50	
165	0	100	
170	0	100	

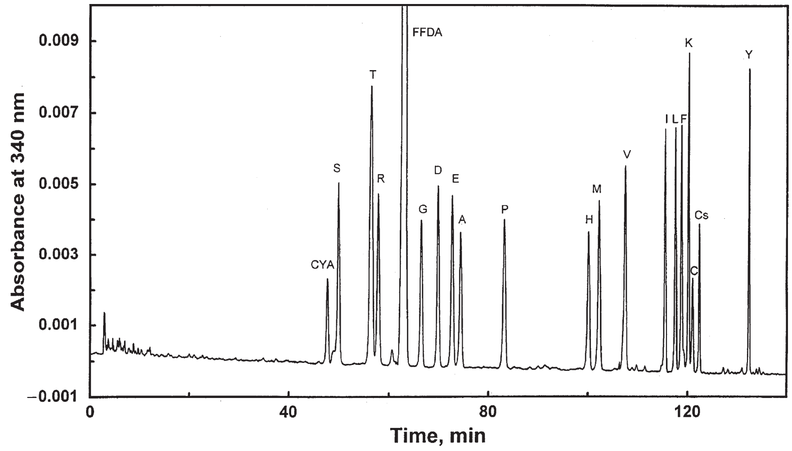

Fig. 2. Separation of 2,4-dinitrophenyl-5-L-alanine amide derivatives of L-amino acids by HPLC. A 20-µL aliquot from the amino acid standard mixture (standard H from Pierce Chemical Company) was derivatized with Marfey's reagent. Chromatographic conditions: Column, LiChrospher 100 RP 8 (250 × 4.6 mm; 5 µm from Macherey-Nagel); sample, 100 pmol of diastereomeric derivatives of 18 L-amino acids; solvent A, 13 m*M* trifluoroacetic acid plus 4% (v/v) tetrahydrofuran in water; solvent B, 50% (v/v) acetonitrile in solvent A; flow rate, 1 mL/min; detection at 340 nm; elution with a linear gradient of solvent B in solvent A (**Table 1**). The amino acid derivatives are denoted by single-letter code, cysteic acid as CYA and cystine as Cs. FFDA indicates the reagent peak; the peaks without denotations are reagent-related as shown in an independent chromatographic run of the reagent alone.

4. Notes

1. For analysis of amino acids as substrates or products in an enzymic reaction, deproteinization is required. Add 4 *M* perchloric acid to a final concentration of 1 *M* and incubate on ice for at least 15 min. Excess perchloric acid is precipitated as $KClO_4$ by adding an equal volume of ice-cold 2 *M* KOH. After centrifugation for 15 min at 4 °C, the supernatant is collected, dried, and used for derivatization.
2. Complete removal of HCl is absolutely essential for quantitative reaction between amino acids and Marfey's reagent.
3. The molar ratio of the Marfey's reagent to the total amino acids should not be more than 3:1.
4. During derivatization, the color of the reaction mixture turns from yellow to orange-red.
5. On acidification, the color of the reaction mixture turns to yellow.
6. The dried amino acids are stable for over one month when stored at −20 °C in the dark. In 50% (v/v) DMSO, derivatives are stable for 72 h at 4 °C and for > 6 wk at −20 °C.
7. To obtain good reproducibility, an HPLC autoinjector is highly recommended.
8. Silica-based C_8 columns from different commercial sources produce baseline resolution of the 19 amino acid derivatives; nevertheless, the gradient slope of solvent B should be optimized for each column. *S*-carboxymethyl-L-cysteine and tryptophan, if included, are also separated in a single chromatographic run (*see* **ref. *2***).
9. The identity of each peak is established by adding a threefold molar excess of the amino acid in question. A reagent blank should be run with each batch of the Marfey's reagent to identify reagent-related peaks. Excess reagent interferes with baseline separation of the arginine and glycine peaks. Lysine and tyrosine are separated as disubstituted derivatives.

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Amino Acid Analysis of Protein Hydrolysates Using Anion Exchange Chromatography and IPAD Detection

Petr Jandik, Jun Cheng, and Nebojsa Avdalovic

1. Introduction

Following the commercial introduction as “AAA Direct,” amino acid analysis by anion exchange chromatography and integrated pulsed amperometric detection (IPAD) ***(1)*** has been applied in many different types of amino acid assays ***(1–16)***. The new method does not require any derivatization. It is thus easier to use and less costly than derivatizaton-based methods. Additional savings are achieved by the relatively inexpensive single-component mobile phases and by the low flow rate (0.25 mL/min).

The limits of detection are in the femtomol range and linear calibration plots extend over three orders of magnitude for the most amino acids ***(1)***.

Various protocols for analyzing protein and peptide hydrolysates are outlined in the literature ***(1,9–11)***. The results obtained for a collagen hydrolysate were compared with those generated by cation exchange / ninhydrin method ***(1)***. Simultaneous analysis of carbohydrates and amino acids was reported by several authors ***(12–16)***. The use of the technique for monitoring and control of metabolism in mammalian cell has been discussed ***(16)***.

In this chapter, we provide suitable protocols for the validation of reproducibility and accuracy. Such validation should be performed not only during a start up but also in regular intervals during the routine use. Evaluation of reproducibility is made easier by a new report format of Dionex software.

We expect many of the new users of AAA Direct to be experienced in one of the post- or pre-column derivatization techniques. In anticipation, we include guidelines for the selection of compatible buffers, surfactants and reagents.

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Several of the compounds that are not compatible or difficult to use with the older methods do not cause any problems when utilized in conjunction with the AAA Direct (i.e. Triton X100, urea, ammonia from the hydrolysis of polyacrylamide, sodium hydroxide). On the other hand, some chemicals that are in widespread use in the pre- or post-column derivatization-based protocols do interfere, if utilized with our method. The most important examples of the latter category are tris(hydroxymethyl)aminomethane (Tris) or carbohydrates when present in high concentrations. However, both Tris and carbohydrates interfere only within the simplest format of AAA Direct. Recently, the authors have reported a modified version of AAA Direct overcoming Tris and carbohydrate interference ***(17,18)***.

2. Materials

2.1. Chromatography

1. Water 18 megohm (Millipore, Bedford, MA, Milli Q or equivalent, *see* **Note 1**)
2. Sodium hydroxide solution 50% w/w (Certified Grade, Fisher Scientific, Pittsburgh, PA)
3. Sodium acetate, anhydrous (Microselect Grade, FLUKA, Milwaukee, MI)
4. Helium or argon gas cylinder, pressure regulation valve, Tygon tubing connecting cylinder to eluent containers.
5. Sterile filtration units from Nalge (0.2 µm Nylon filter in covered funnels, vol = 1 L, VWR, West Chester, PA)
6. Serological glass pipets, vol =10 mL (Fisher Scientific)
7. Eluent A: Water 18 megohm filtered through a 0.2 µm Nylon filter (*see* **Notes 2** and **3**)
8. Eluent B: Filter 1 L of water through a 0.2 µm Nylon filter; using the serological pipets (see **Note 4**), add 13.1 mL of 50% sodium hydroxide to the filtered 1L-volume of water (see **Note 3**).
9. Eluent C: Dissolve 82 g of anhydrous sodium acetate in ca. 500 mL of water. Fill up to 1L and filter through the 0.2 µm Nylon filter.
10. AminoPac PA10 Guard Column (2 × 50mm) and AminoPac PA10 analytical column (2 × 250mm).
11. Ternary low-pressure gradient, microbore, HPLC system, Dionex BioLC or comparable with an autosampler (e.g. Dionex AS50) and column thermostat. The system must include Dionex ED40 electrochemical detector.
12. AAA certified gold cell for use with the ED40 detector.
13. PC with installed copy of Dionex PeakNet 6.1 or equivalent (*see* **Note 5**).

2.2. Reproducibility Testing Using Injections of Standards

1. Amino Acids in 0.1 *M* HCl, Standard Reference Material 2389 (National Institute of Standards and Technology, Gaithersburg, MD)
2. Norleucine (Sigma, St. Louis, MO)
3. Sodium azide (Sigma)
4. 0.1 *M* HCl (reagent grade)

5. Diluent containing norleucine and sodium azide: Prepare 4 m*M* stock solution of norleucine (524.8 mg/L) in 0.1 *M* HCl. Dilute 500x with a solution of azide (20mg/L).
6. Reproducibility Standard: pipet 320 µL of SRM 2389 into a 100 mL volumetric flask and fill up to volume with the norleucine diluent. If stored in a refrigerator, the Reproducibility Standard is stable for up to 30 days. If used at room temperature, the Reproducibility Standard remains stable for up to three days (i.e. over a weekend).
7. The Reproducibility Standard from **item 6** does not contain all of the standard compounds that are required for the analysis of certain types of hydrolysate samples. These standards can be purchased from Sigma and are listed below: δ-hydroxylysine, D(+)-galactosamine hydrochloride, D(+)-glucosamine hydrochloride, hydroxy-L-proline, phospho-L-arginine sodium salt, o-phospho-D,L-serine, o-phospho-D,L-threonine, o-phospho-D,L-tyrosine, L-cysteic acid hydrate and L-tryptophan.
8. Prepare 1 mM, single component solutions of all standards from step 7 in 0.1 M HCl and store them in a refrigerator. As required, add 100 µL of one or more of the above single-component stock solution to the Reproducibility Standard (step 6).
9. Disposable transfer pipets (glass or polyethylene)

2.3. Accuracy of Amino Acid Analysis in a BSA Hydrolysate

1. Bovine serum albumin (7% solution) Standard Reference Material 927c (National Institute of Standards and Technology, Gaithersburg, MD)
2. Hydrolysate glass tubes with Teflon Plugs (Pierce, Rockford, IL)
3. Speedvac (Savant, Farmingdale, NY)
4. Heating Module with aluminum heating block (Pierce)
5. Constant Boiling HCl (Pierce)

2.4. Selecting Suitable Buffers, Surfactants and Reagents (see Note 6*)*

1. Buffers, surfactants, and reagents found to be compatible with AAA Direct: ACES (*see* **Note 7**); ADA; AMPSO; CAPS (*see* **Note 7**); CAPSO; CHES (*see* **Note 7**); citric acid (Fluka); EDTA (Aldrich); MES (Boehringer Manheim); MOBS; MOPS; MOPSO; n-octyl-β-glucoside (*see* **Note 8**); phenol; PIPES (*see* **Note 7**) sodium diphosphate; sodium monophosphate; sodium triphosphate; sodium azide; TES; TRITON X 100 (Aldrich).
2. Buffers, surfactants, and reagents producing *interfering* peaks in AAA Direct: BES (BDH Chemicals, Poole England); Bicine; BISTRIS (*see* **Note 9**); CHAPS; CHAPSO; DTE; EPPS; HEPES; imidazole; TEA (*see* **Note 8**); Tricine (*see* **Note 9**); TRIS (*see* **Note 9**).

3. Methods

3.1. Chromatography (see Note 10*)*

The chromatogram in **Fig. 1** was generated using the gradient conditions in **Table 1** and detection conditions in **Table 2**. We recommend using those conditions for all reproducibility and accuracy testing described below.

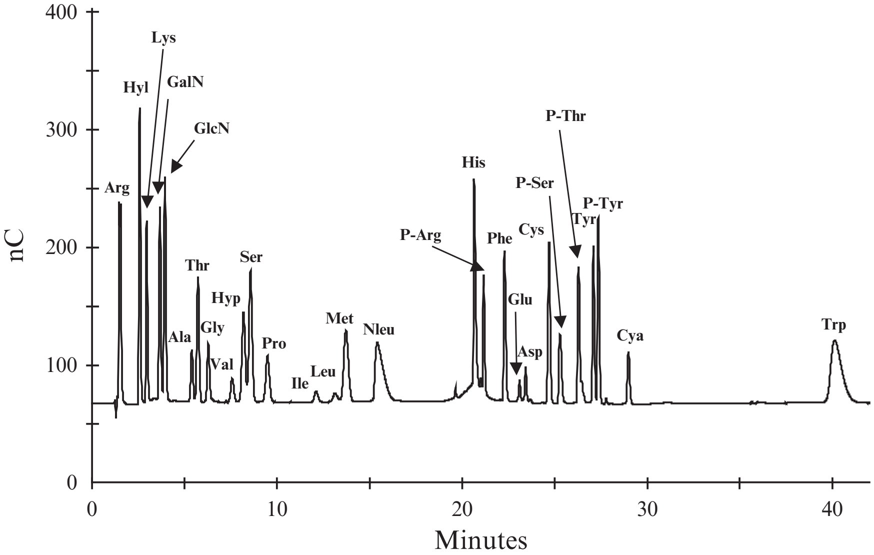


Fig. 1. Simultaneous separation of amino acids (including hydroxy-proline and hydroxylysine, or Hyp and Hyl, respectively), amino sugars (GalN and GlcN), phospho amino acids (P-Arg, P-Ser and P-Thr) and cysteic acid (Cya) under the conditions of standard AAA Direct gradient.

Fig. 1 illustrates the capability of AAA Direct for protein and peptide hydrolysates. All common types of hydrolysates (HCl, HCl /propionic acid, MSA, NaOH,) can be analyzed under identical gradient and detection conditions. An important exception is the separation of methionine sulfone, that can be achieved only at the column temperature of 35 °C (See **ref *9***). In contrast, the correct column temperature for all other separations (including that in **Fig. 1**) is 30 °C.

1. Connect the fully assembled ED40 cell directly to the injector outlet. Running the initial gradient conditions (76%A, 24%B at 0.25 mL/min) switch the cell voltage on (Specify "Integrated Amperometry" and apply Waveform from **Table 2**). Verify that the signal is lower than 80 nC.
2. Install the AminoPac PA10 column set and check again that the signal background is at less than 80 nC.
3. Allow the column oven to reach 30 °C and run a blank gradient (*see* **Table 1**) injecting 25 µL of water. Verify that gradient rise is not exceeding 30 nC.
4. Prepare an aliquot of Reproducibility Standard (**Subheading 2.2, item 6**) and place it into the autosampler. Follow proper procedures in executing the first standard injection (vol = 25 µL) by not allowing any delay between the end of the blank gradient run and first injection of standard.
5. Verify the absence of extraneous peaks in the blank gradient and a correct value of peak height for arginine (>100 nC). Verify the presence of peaks for all amino

acids. P-amino acids, hydroxylysine, hydroxyproline, amino sugars, cysteic acid and tryptophan are not present in the Reproducibilty Standard. Verify that the separation between alanine and threonine in the Reproducibility Standard chromatogram is comparable to that in **Fig. 1**.

3.2. Reproducibility Testing Using Injections of Standards

1. Verify the system status by carrying out steps 1-5, **Subheading 3.1**.
2. Transfer 1.5 mL of Reproducibility Standard (**Subheading 2.2, step 6**) into a glass autosampler vial. Place the full vial into the autosampler. Make sure there is also a vial with water inside the autosampler.
3. Create a "PeakNet Sequence" for running a blank gradient (vol = 25 µL injection of water, **Table 1** gradient, **Table 2** waveform) followed by nine injections of Reproducibility Standard (vol = 25 µL "Full Loop," **Table 1, Table 2**). Do not perform more than nine vol =25 µL Full-Loop-injections out of a single 1.5 mL-vial.
4. Create or update a quantitation method (.qnt) and include it in the PeakNet sequence. A meaningful evaluation of reproducibility relies on correct identification and integration of all peaks of interest. The correct identification and integration is dependent on a correct user input of retention times and integration parameters (*see* **Note 11**). The retention times can be updated and integration parameters optimized during the

Table 1
Gradient Conditions for Hydrolysates (Flow rate: 0.25 mL/min)

Time (min)	%A*	%B*	%C*	Curve
Init.	76	24	0	
0.00	76	24	0	
2	76	24	0	
8	64	36	0	8
11	64	36	0	
18	40	20	40	8
21	44	16	40	5
23	14	16	70	8
42	14	16	70	
42.1	20	80	0	5
44.1	20	80	0	
44.2**	76	24	0	5
75***	76	24	0	

* Eluents A,B,C: See Subheading 2.1., Steps7,8,9

**Start of reequilibration to initial conditions

***Complete reequilibration and column clean-up under normal conditions

Table 2
Detection Conditions

Time (milli seconds)	Potential (V)* vs. pH	Integration
0	0.13	
40	0.13	
50	0.33	
110	0.33	
210	0.33	Begin
220	0.55	
460	0.55	
470	0.33	
560	0.33	End
570	−1.67	
580	−1.67	
590	0.93	
600	0.13	

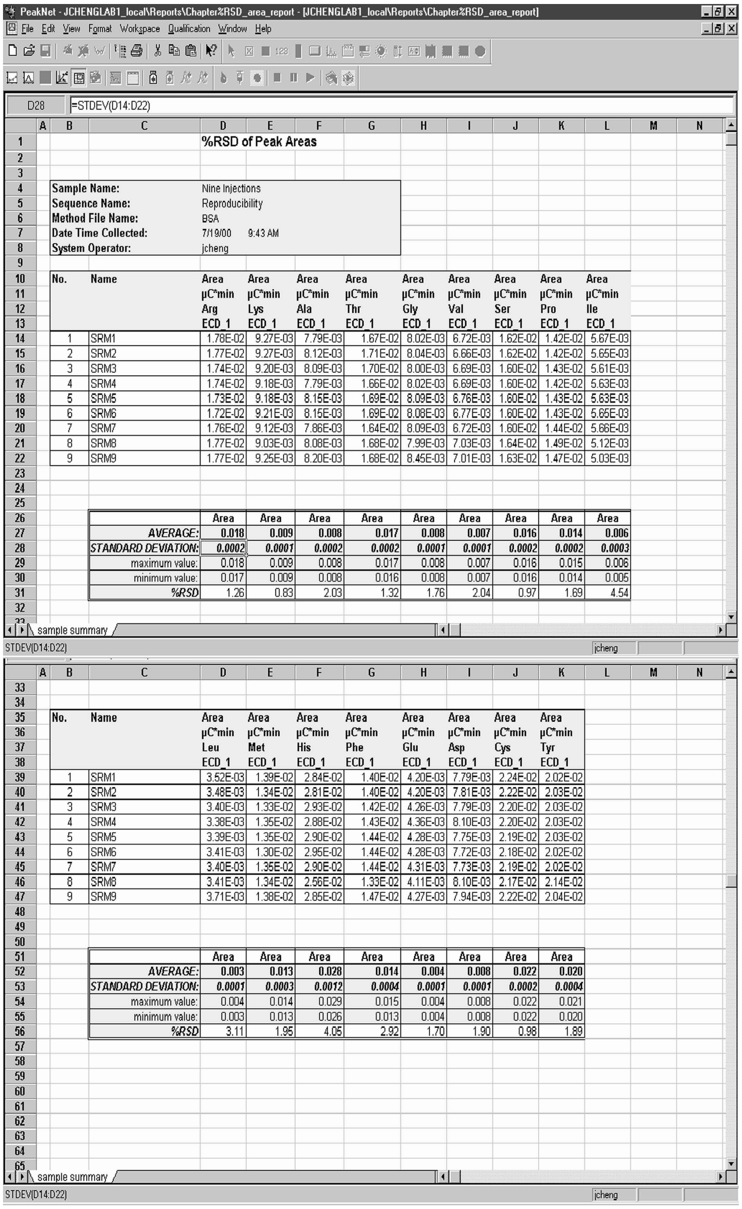
*Sequence of potentials (or waveform) applied to the Au working electrode and referenced vs. Glass/Ag/AgCl combination electrode.

first standard run. It is not necessary to input pmol amounts for amino acids and to carry out a calibration at this stage as reproducibility is evaluated from the relative standard deviations of peak areas.

5. Following the completion of all standard runs, verify correct identification and integration of all peaks by inspecting all nine standard chromatograms separately. Close the files you opened for identification and integration verification.
6. Select all nine standard chromatograms simultaneously (press Ctrl. and click on corresponding row numbers). In the menu that opens up by rightclicking on one of the selected rows, choose "compare>ECD1" and an overlay of all selected chromatograms appears on the screen.
7. Rightclick anywhere in the upper-right portion of the chromatographic overlay. In the menu that now appears choose "Load Report Format>%RSD Peak Areas" (*see* **Note 12**) to generate a similar report as the one shown in **Fig. 2**.
8. Rightclick in the upper-right portion of the screen, go to "Load Report Format" again and switch to %RSD Retention Times (actual report not shown).

3.3. Accuracy of Amino Acid Analysis in a BSA Hydrolysate

The following protocol utilizes a data reduction procedure producing three numerical QC values, "Average % Error," Recovery [g],"and "% Recovery," characterizing the accuracy of results for a protein of known amino acid composition.


PeakNet - JCHENGLAB1_local\Reports\Chapter%RSD_area_report - [JCHENGLAB1_local\Reports\Chapter%RSD_area_report]
File Edit View Format Workspace Qualification Window Help
D28
sample summary
STDEV(D14:D22)
jcheng

=STDEV(D14:D22)

%RSD of Peak Areas

Sample Name: Nine Injections
Sequence Name: Reproducibility
Method File Name: BSA
Date Time Collected: 7/19/00 9:43 AM
System Operator: jcheng

No.	Name	Area µC*min Arg ECD_1	Area µC*min Lys ECD_1	Area µC*min Ala ECD_1	Area µC*min Thr ECD_1	Area µC*min Gly ECD_1	Area µC*min Val ECD_1	Area µC*min Ser ECD_1	Area µC*min Pro ECD_1	Area µC*min Ile ECD_1
1	SRM1	1.78E-02	9.27E-03	7.79E-03	1.67E-02	8.02E-03	6.72E-03	1.62E-02	1.42E-02	5.67E-03
2	SRM2	1.77E-02	9.27E-03	8.12E-03	1.71E-02	8.04E-03	6.66E-03	1.62E-02	1.42E-02	5.65E-03
3	SRM3	1.74E-02	9.20E-03	8.09E-03	1.70E-02	8.00E-03	6.69E-03	1.60E-02	1.43E-02	5.61E-03
4	SRM4	1.74E-02	9.18E-03	7.79E-03	1.66E-02	8.02E-03	6.69E-03	1.60E-02	1.42E-02	5.63E-03
5	SRM5	1.73E-02	9.18E-03	8.15E-03	1.69E-02	8.09E-03	6.76E-03	1.60E-02	1.43E-02	5.63E-03
6	SRM6	1.72E-02	9.21E-03	8.15E-03	1.69E-02	8.08E-03	6.77E-03	1.60E-02	1.43E-02	5.65E-03
7	SRM7	1.76E-02	9.12E-03	7.86E-03	1.64E-02	8.09E-03	6.72E-03	1.60E-02	1.44E-02	5.66E-03
8	SRM8	1.77E-02	9.03E-03	8.08E-03	1.68E-02	7.99E-03	7.03E-03	1.64E-02	1.49E-02	5.12E-03
9	SRM9	1.77E-02	9.25E-03	8.20E-03	1.68E-02	8.45E-03	7.01E-03	1.63E-02	1.47E-02	5.03E-03

	Area	Area	Area	Area	Area	Area	Area	Area	Area
AVERAGE:	**0.018**	**0.009**	**0.008**	**0.017**	**0.008**	**0.007**	**0.016**	**0.014**	**0.006**
STANDARD DEVIATION:	***0.0002***	***0.0001***	***0.0002***	***0.0002***	***0.0001***	***0.0001***	***0.0002***	***0.0002***	***0.0003***
maximum value:	0.018	0.009	0.008	0.017	0.008	0.007	0.016	0.015	0.006
minimum value:	0.017	0.009	0.008	0.016	0.008	0.007	0.016	0.014	0.005
%RSD	1.26	0.83	2.03	1.32	1.76	2.04	0.97	1.69	4.54

No.	Name	Area µC*min Leu ECD_1	Area µC*min Met ECD_1	Area µC*min His ECD_1	Area µC*min Phe ECD_1	Area µC*min Glu ECD_1	Area µC*min Asp ECD_1	Area µC*min Cys ECD_1	Area µC*min Tyr ECD_1
1	SRM1	3.52E-03	1.39E-02	2.84E-02	1.40E-02	4.20E-03	7.79E-03	2.24E-02	2.02E-02
2	SRM2	3.48E-03	1.34E-02	2.81E-02	1.40E-02	4.20E-03	7.81E-03	2.22E-02	2.03E-02
3	SRM3	3.40E-03	1.33E-02	2.93E-02	1.42E-02	4.26E-03	7.79E-03	2.20E-02	2.03E-02
4	SRM4	3.38E-03	1.35E-02	2.88E-02	1.43E-02	4.36E-03	8.10E-03	2.20E-02	2.03E-02
5	SRM5	3.39E-03	1.35E-02	2.90E-02	1.44E-02	4.28E-03	7.75E-03	2.19E-02	2.03E-02
6	SRM6	3.41E-03	1.30E-02	2.95E-02	1.44E-02	4.28E-03	7.72E-03	2.18E-02	2.02E-02
7	SRM7	3.40E-03	1.35E-02	2.90E-02	1.44E-02	4.31E-03	7.73E-03	2.19E-02	2.02E-02
8	SRM8	3.41E-03	1.34E-02	2.56E-02	1.33E-02	4.11E-03	8.10E-03	2.17E-02	2.14E-02
9	SRM9	3.71E-03	1.38E-02	2.85E-02	1.47E-02	4.27E-03	7.94E-03	2.22E-02	2.04E-02

	Area	Area	Area	Area	Area	Area	Area	Area
AVERAGE:	**0.003**	**0.013**	**0.028**	**0.014**	**0.004**	**0.008**	**0.022**	**0.020**
STANDARD DEVIATION:	***0.0001***	***0.0003***	***0.0012***	***0.0004***	***0.0001***	***0.0001***	***0.0002***	***0.0004***
maximum value:	0.004	0.014	0.029	0.015	0.004	0.008	0.022	0.021
minimum value:	0.003	0.013	0.026	0.013	0.004	0.008	0.022	0.020
%RSD	3.11	1.95	4.05	2.92	1.70	1.90	0.98	1.89

Fig. 2. Reproducibility Report. Relative standard deviations for each amino acid are listed in the bottom row. The report is linked to all data files in a given PeakNet Sequence. Any change of integration parameters produces instant updates of the corresponding area, average, and %RSD values.

$$\textbf{Average \% Error} = (\Sigma\ \%\ \textit{Error} \text{ of 16 amino acids}) / 16 \tag{1}$$

Where:
% Error = 100* ABS (analytical result for *n* – true value for *n*)/true value for *n*
ABS (x): absolute value of x
n = number of each amino acid per molecule

$$\textbf{Recovery [g]} = \{(\textit{Corrected Average Ratio})\ ^{*} \text{ molecular weight of protein}\} \tag{2}$$

Where:
Corrected Average Ratio: mean value of those *First Ratios* values deviating by less than 20% from the Average of *First Ratios* (*Corrected Average Ratio* represents the analytical result for the mol-amount of protein injected)
First Ratio = analytical result in pmol of amino acid/ true value of *n*

$$\textbf{\% Recovery} = \{(\textbf{Recovery [g]}\ ^{*}\textbf{8}\}/ \text{ g of Protein in } 200\,\mu\text{L of hydrolysate}) \tag{3}$$

This data reduction approach has been used, for example, in the "multicenter" (round robin) studies organized by the Association of Biomolecular Resource Facilities or ABRF *(19)*. Evaluation of accuracy is simplified in AAA Direct by an automatic calculation of QC parameters from pmol results after calibration. As in the reproducibility evaluation (**Subheading 3.2**), the parameters are cal culated directly within the HPLC software. It is not necessary to export area or pmol results into another application such as Excel first (*see* **Note 14**).

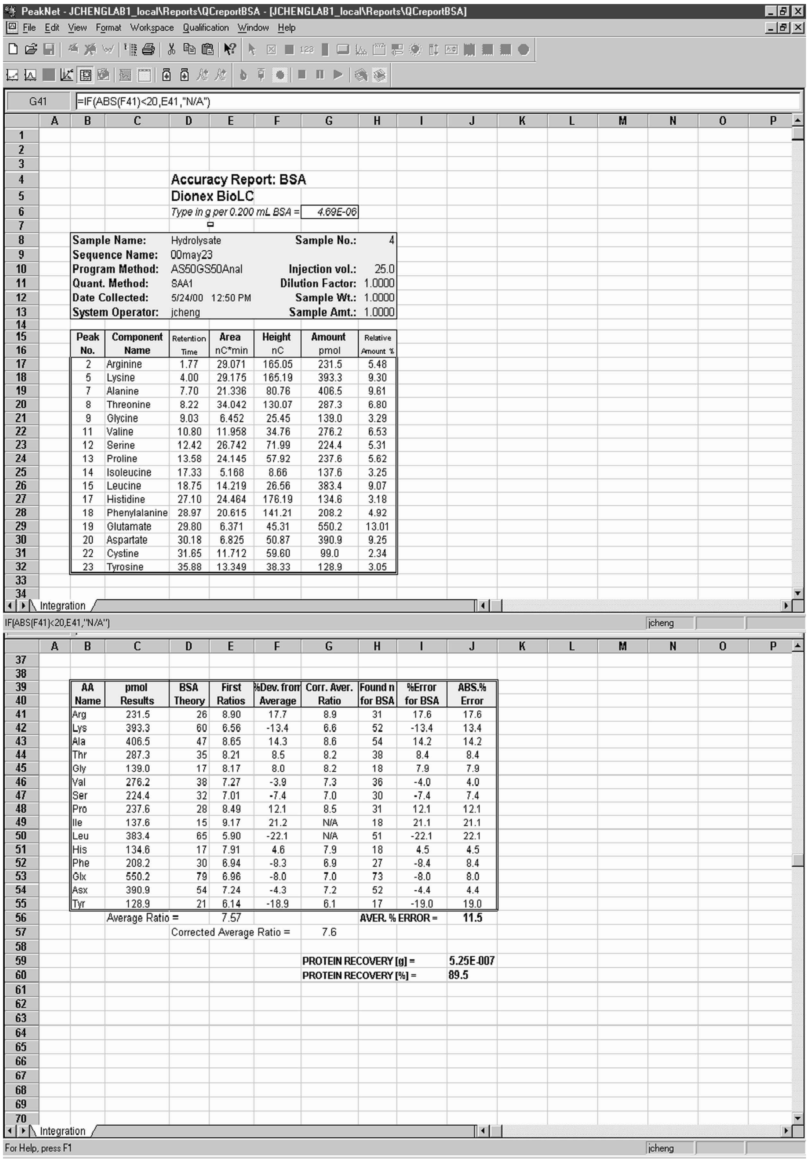
As a protein of known amino acid composition, we have chosen the 7 % BSA solution from the National Institute of Standard and Technology (**Subheading 2.3**, **item 1**). The amino acid composition of BSA can be obtained in the literature *(20)*. The NIST documentation certifies that the SRM 927c is from bovine blood and has the correct molecular weight and spectral properties for BSA. It also states that the SRM 927c may be used for calibration in amino acid analysis. The SRM 927c documentation, however, does not certify the amino acid composition of the actual protein sample used to prepare the 7% solution.

1. Prepare a 500fold dilution of SRM 927c in water containing 20 mg/L sodium azide.
2. Transfer 33.6 µL of 500x dilute SRM 927c into a clean microvial (vol=1.5 mL) and carry out evaporative centrifugation (Speedvac) to dryness.
3. Add to the dry residue 100 µL of constant boiling HCl and vortex thoroughly.
4. Using glass Pasteur pipets transfer the solution to a clean hydrolysis tube (**Subheading 2.3, step 2**).
5. To the microvial emptied in step 4, add a new 50 µL aliquot of constant boiling HCl, vortex and subsequently transfer as much as possible of the 50 µL aliquot to the sample in the hydrolysis tube from step 4. Use the same transfer pipet as in **step 4**.

6. Alternate vacuum and inert gas inside the hydrolysis tube three times. Place fully evacuated tube into a heating module (**Subheading 2.3, item 4**). Carry out hydrolysis for 16–24 hours at 110 °C.
7. Remove the hydrolysis tube from the heating module and allow it to cool down to the ambient temperature.
8. Using a clean glass Pasteur pipet transfer the hydrolyzed sample from the hydrolysis tube into a clean microvial.
9. Pipet another 50 μL aliquot of constant boiling HCl into the hydrolysis tube from **step 8**. Rinse and subsequently transfer as much as possible of the 50 μL aliquot to the first aliquot of hydrolysate in the microvial. Use the same transfer pipet for steps 8 and 9.
10. Carry out evaporative centrifugation of the combined hydrolysate aliquots from steps 8 and 9.
11. To the completely dry residue from step 10, add 200 μL of norleucine/azide diluent (**Subheading 2.2, item 5**) and vortex thoroughly.
12. Transfer an aliqot of the reconstituted hydrolysate into an autosampler vial (Polyethylene vial, (vol=0.3 mL, with a split septum, Dionex). Make sure that vials with water and calibration standard have also been placed into the autosampler tray.
13. Create a "PeakNet Sequence" starting with an injection of water blank and followed by at least three injections of both, the calibration standard (**Subheading 2.2, item 6**) and hydrolyzed sample. Use a program file (.pgm) that includes the gradient and waveform from **Tables 1** and **2**, respectively. Input correct pmol amounts (*see* **Note 15**) into the Amount Table of the quantitation method (.qnt, **Subheading 3.2, step 4**). Use 25 μL Fool-Loop injections for all samples and standards. In the quantitation method, specify the sequence line containing the calibration standard. Start the automatic execution of the entire sequence.
14. After executing all runs, inspect each standard and sample chromatogram. Verify correct identification and integration of all amino acid peaks.

In a hydrolysate chromatogram, rightclick in the upper-right portion of the chromatogram. In the menu that now appears choose "Load Report Format>QCBSA (see **Note 14**)." A similar report as the one in **Fig. 3** appears on the screen.

15. Make sure to input the actual concentration of BSA in the hydrolysate as specified in the report subtitle (line 3). Switch to "Layout Mode" (Edit>Layout Mode) to enable input of BSA concentration. The calculated values of Average % Error, Protein Recovery [g] and % Protein Recovery appear instantaneously in the lower half of the report page. The BSA QC report is now ready to be saved or printed out.
16. Additional QC Reports for the other BSA hydrolysate injections can be generated by moving from one sample file to another using the corresponding two icons on the screen (left and right arrow, top bar, third row). To complete the documentation, it is also possible to generate the %RSD Area , %RSD Retention Times reports for the standard injections of the same sequence by carrying out the steps 6 to 8 of the **Subheading 3.2.**


PeakNet - JCHENGLAB1_local\Reports\QCreportBSA - [JCHENGLAB1_local\Reports\QCreportBSA]
File
Edit
View
Format
Workspace
Qualification
Window
Help
Integration
jcheng
For Help, press F1

G41 =IF(ABS(F41)<20,E41,"N/A")

Accuracy Report: BSA

Dionex BioLC

Type in g per 0.200 mL BSA = 4.69E-06

Sample Name:	Hydrolysate	**Sample No.:**	4
Sequence Name:	00may23		
Program Method:	AS50GS50Anal	**Injection vol.:**	25.0
Quant. Method:	SAA1	**Dilution Factor:**	1.0000
Date Collected:	5/24/00 12:50 PM	**Sample Wt.:**	1.0000
System Operator:	jcheng	**Sample Amt.:**	1.0000

Peak No.	Component Name	Retention Time	Area nC*min	Height nC	Amount pmol	Relative Amount %
2	Arginine	1.77	29.071	165.05	231.5	5.48
5	Lysine	4.00	29.175	165.19	393.3	9.30
7	Alanine	7.70	21.336	80.76	406.5	9.61
8	Threonine	8.22	34.042	130.07	287.3	6.80
9	Glycine	9.03	6.452	25.45	139.0	3.29
11	Valine	10.80	11.958	34.76	276.2	6.53
12	Serine	12.42	26.742	71.99	224.4	5.31
13	Proline	13.58	24.145	57.92	237.6	5.62
14	Isoleucine	17.33	5.168	8.66	137.6	3.25
15	Leucine	18.75	14.219	26.56	383.4	9.07
17	Histidine	27.10	24.464	176.19	134.6	3.18
18	Phenylalanine	28.97	20.615	141.21	208.2	4.92
19	Glutamate	29.80	6.371	45.31	550.2	13.01
20	Aspartate	30.18	6.825	50.87	390.9	9.25
22	Cystine	31.65	11.712	59.60	99.0	2.34
23	Tyrosine	35.88	13.349	38.33	128.9	3.05

IF(ABS(F41)<20,E41,"N/A")

AA Name	pmol Results	BSA Theory	First Ratios	%Dev. from Average	Corr. Aver. Ratio	Found n for BSA	%Error for BSA	ABS.% Error
Arg	231.5	26	8.90	17.7	8.9	31	17.6	17.6
Lys	393.3	60	6.56	-13.4	6.6	52	-13.4	13.4
Ala	406.5	47	8.65	14.3	8.6	54	14.2	14.2
Thr	287.3	35	8.21	8.5	8.2	38	8.4	8.4
Gly	139.0	17	8.17	8.0	8.2	18	7.9	7.9
Val	276.2	38	7.27	-3.9	7.3	36	-4.0	4.0
Ser	224.4	32	7.01	-7.4	7.0	30	-7.4	7.4
Pro	237.6	28	8.49	12.1	8.5	31	12.1	12.1
Ile	137.6	15	9.17	21.2	N/A	18	21.1	21.1
Leu	383.4	65	5.90	-22.1	N/A	51	-22.1	22.1
His	134.6	17	7.91	4.6	7.9	18	4.5	4.5
Phe	208.2	30	6.94	-8.3	6.9	27	-8.4	8.4
Glx	550.2	79	6.96	-8.0	7.0	73	-8.0	8.0
Asx	390.9	54	7.24	-4.3	7.2	52	-4.4	4.4
Tyr	128.9	21	6.14	-18.9	6.1	17	-19.0	19.0

Average Ratio = 7.57

AVER. % ERROR = **11.5**

Corrected Average Ratio = 7.6

PROTEIN RECOVERY [g] = **5.25E-007**

PROTEIN RECOVERY [%] = **89.5**

Fig. 3. Accuracy Report. The upper table is the usual "Amount Table" of PeakNet HPLC software. The pmol amounts in the sample are calculated from the pmol/area coefficients in the calibration file. The lower of the two tables evaluates equations (***1–3***) in the **Subheading 3.3**. This report is also linked to all data files of a given sequence. Any change of integration parameters produces an instant change of all calculated results.

3.4. Selecting Suitable Buffers, Surfactants, and Reagents

1. Generate chromatograms of 1 m*M* solutions of all buffers, surfactants and reagents present in your protein or peptide samples.
2. Hydrolyse (**Subheading 3.3.,** steps 6–12) 20 µL aliquots of 10 mM solutions of all buffers, surfactants and reagents in 180 µL of constant boiling HCl.
3. Generate chromatograms of hydrolyzed and reconstituted (azide/norleucine diluent) samples from steps 1 and 2 using the chromatographic conditions of **Subheadings 3.2 and 3.3**.
4. Avoid using chemicals causing peaks interfering with amino acids or carry out desalting prior to hydrolysis of protein solutions containing such additives. See **Subheading 2.4**. for guidelines in the selection of new additives.
5. Repeat steps 1-4 for all new batches of additives.

4. Notes

1. Minimize extraneous plastic tubing connected to the laboratory water unit and use only filters supplied by the manufacturers. Polymeric surfaces support bacterial growth. Bacterial contamination of water used in preparation of eluents causes high backgrounds and spurious peaks in amino acid analysis.
2. The main purpose of eluent filtration is to eliminate bacterial contamination stemming from the water purification unit and from the atmosphere. Wear powder-free gloves whenever preparing or replenishing mobile phases to avoid contamination by skin contact.
3. Minimize carbonate contamination of the eluents. Follow the guidelines in the Installation Instructions and Troubleshooting Guide for AAA Direct Amino Acid Analysis System (Dionex Document No. 031481).
4. Most vol=10 mL pipets can be used for the prescribed volume of 13.1 mL. Usually this larger volume is at or near the markings for the maximum possible volume. Verify the correct height of 13.1 mL by pipeting and weighing out water. Do not re-use serological pipets.
5. Make sure that the "Report Publisher" option is part of the installed software and it is switched on. (In the Pull-down menu: Help/About PeakNet)
6. Except where indicated otherwise, all of the buffers, reagents and surfactants were purchased from Sigma.
7. Four of the buffers that we evaluated (ACES, CAPS, CHES, PIPES) gave well-defined peaks, sufficiently separated from those of hydrolysate amino acids under the gradient conditions in **Table 1**. Users are advised to run control blanks when planning to utilize those buffers. Some of the peaks appear to be impurities unrelated to the actual buffer compound and may vary depending on the source and age of the chemical.
8. Glucoside-based surfactants produce a large interference peak when injected directly. However, they are completely hydrolyzed after 16–24 hours and

110 °C in constant boiling HCl and do not thus interfere in a completely hydrolysed sample.

9. Several of the non-anionic buffers (BISTRIS, TEA, TricineTRIS) produce a large peak interfering with arginine. This interference can be resolved using the modified AAA Direct technique described in **ref.** ***(17)***.
10. A copy of the Installation Instructions and Troubleshooting Guide for the AAA Direct Amino Acid Analysis (Dionex Document No. 031481) is included with each AminoPac PA10 column shipped by Dionex. The text under Heading 3 describes only a subset of AAA Direct methodology used for reproducibility and accuracy testing.
11. Optimization of integration parameters and other general guidelines for reproducible quantitation are described in "PekNet 6 User's Guide." (Dionex Document No. 031630). Specify the version of PeakNet you are running when ordering the document from Dionex.
12. The "Excel-like" environment in the report part of Dionex HPLC software makes it possible for a user to create many different report formats executing simple or complex mathematical formulas. However, the user has to purchase the "Report Publisher" option to be able to generate a custom report format (*see* **Note 5** for verification of presence of Report Publisher in the installed software). The electronic files for reproducibility evaluation without installing the Report Publisher can be obtained from Dionex Customer Support. Specify "Reproducibility Evaluation of AAA Direct" when ordering the report format file.
13. A comprehensive evaluation of AAA Direct in conjunction with hydrolysis of peptides in HCl and MSA is presented in **ref.** ***(10)***. **Reference** ***(9)*** contains detailed protocols for HCl, MSA (with and without performic acid oxidation) and NaOH hydrolysates. Also described in **ref.** ***(9)*** is a new method for using the same set of chromatographic conditions for automatic analysis of carbohydrates, amino sugars and amino acids in different types of TFA and HCl hydrolysates of glycoproteins.
14. As in the reproducibility evaluation (**Subheading 3.2** and **Note 12**), it is possible for a user to create highly customized report formats for the QC parameters of accuracy evaluation. The user has to have either the "Report Publisher" option installed on the PC (see **Note 5**) or obtain a suitable report format file from Dionex customer support. Specify "Accuracy of Amino Acid Analysis" when ordering the report format file from Dionex.
15. For a 321.5-fold dilution of SRM 2389 and 25 µL injection, divide all values in **Table 1** of SRM 2389 by 0.0125. For example: the original concentration of 2.50 mmol/L of aspartic acid yields 200 pmol amount to be entered into the Amount Table.

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Validation of Amino Acid Analysis Methods

Andrew J. Reason

1. Introduction (*see* Notes 1–4)

This chapter presents a discussion of the points to consider during the validation of analytical methods and more specifically amino acid analysis. Amino acid analyses are utilised in various areas of research for free amino acids (in, for example, foodstuffs) and for analysis of products and components of interest comprising peptides, polypeptides and peptide/protein conjugates. In this Chapter, I have concentrated on the validation of amino acid analyses that are to be submitted in a package to obtain a regulatory authority licence for a peptide, protein, or conjugate product. In such studies, amino acid analyses can be used to provide various pieces of information generally required for biotechnological and/or biological products. For example quantitative amino acid analysis can be used to determine protein quality and quantity. Such data, combined with Optical Density (OD) measurements, can be used to determine the extinction coefficient for a protein.

Analyses that are to be submitted in a package to obtain a regulatory authority licence for a particular product should be validated in accordance with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Harmonised Tripartite Guidelines "Validation of Analytical Procedures: Text and Methodology" ***(1)*** except where there are specific issues for unique tests used for analyzing biotechnological and biological products ***(2)***. The terms and definitions within these documents are meant to bridge the differences that often exist between the regulators in Europe, Japan and USA. The validation criteria discussed below can be assessed using both precolumn and postcolumn amino acid analysis derivatization methods. The available techniques are described in detail in other chapters of this book ***(10–15)***. Requirements of speed and sensitivity have led

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M-Scan to utilize a method involving acid hydrolysis followed by precolumn derivatization using phenylisothiocyanate and separation by reverse-phase high-performance liquid chromatography (RP-HPLC) for amino acid analysis. This method is used as a background to the discussion of validation of amino acid analysis methods.

2. Materials

2.1. Equipment for Hydrolysis of Proteins and Precolumn Derivatization of the Released Amino Acids (See Note 5)

1. An RP-HPLC system equipped with a Supelcosil LC-18-DB (250 � 4.6 mm, 5 µm; Supelco, Poole, UK) HPLC column, and capable of tertiary solvent/buffer supply, gradient control, autoinjection, UV detection at 254 nm and data acquisition (*see* **Note 6**).
2. Vacuum hydrolysis tubes (Pierce, Rockford, IL) 5 mL internal volume.
3. Vacuum pump system capable of evacuating hydrolysis tubes to 1–2 Torr.
4. Oven or dry heating block capable of heating vacuum hydrolysis tubes to hydrolysis temperature (usually 110°C).

2.2. Chemicals (see Note 5)

2.2.1. Hydrolysis of Proteins and for Precolumn Derivatization of the Released Amino Acids

1. Constant boiling 6 *M* HCl (Pierce).
2. Coupling buffer, acetonitrile:pyridine:water:triethylamine (10:5:3:2 v/v).
3. Phenylisothiocyanate (Pierce).
4. Amino Acid Standard H (Pierce), containing a mixture of 17 amino acids at 2.5 m*M* (cystine at 1.25 *M*) in 0.1 *M* HC1 (*see* **Note 7**).
5. Internal standard, L-Norleucine (Pierce).

2.2.2. RP-HPLC Separationof Derivatized Amino Acids

1. Solvent A: Ultra High Quality (UHQ) water (1 L).
2. Solvent B: Acetonitrile (HPLC grade):UHQ water (80:20 v/v), 1 L.
3. Buffer C, Weigh out 28.7g of anhydrous sodium acetate and dissolve in 500 mL of UHQ water ensuring that all the solid dissolves. Add 1.25 mL of triethylamine and adjust the pH of the buffer to 6.4 using glacial acetic acid.
4. Sample loading solvent, Solvent A:Solvent B:Buffer C (75:5:20 v/v).

3. Methods

3.1. Preparation of Amino Acid Standard Mixture

1. Weigh out a known amount of norleucine internal standard (~1 mg).
2. Dissolve the weighed internal standard (Norleucine) in 5% (v/v) acetic acid in water to generate a 1 nanomole/µL solution. This solution is stable for up to 12 mo at −20° C ± 5°C and can be used as the internal standard bulk solution.

3. An aliquot (50 µL) of the resulting solution, i.e., 50 nmoles is added to each sample being prepared for amino acid analysis and to the tube to be used for the amino acid standard mixture.
4. Add 20 µL of Amino Acid Standard H to 50nmoles of the norleucine (internal standard) in the tube set aside for the standard mixture.
5. The standard mixture of amino acids including the internal standard (norleucine) is derivatized in parallel with the samples to be studied (*see* **Subheading 3.3**).

3.2. Hydrolysis (see Notes 8 and 9)

1. Aliquot the desired amount of peptide, protein or conjugate (ideally 50–100 µg) to be hydrolyzed into a clean vacuum hydrolysis tube and label the tube(s) to identify the sample.
2. Add a known amount (ideally 50 nmoles) of norleucine (internal standard) to the sample tube.
3. Lyophilize resulting sample under vacuum using a Savant or similar drying device.
4. Add 200 µL of 6 *M* (constant boiling) hydrochloric acid.
5. Evacuate the vacuum hydolysis tube(s) for 2 min with agitation.
6. Incubate samples in a heating block or oven at 110°C for 24 h.
7. Allow samples to cool and lyophilize the products.
8. Derivatize released amino acids with phenylisothiocyanate.

3.3. Derivatization of Released Amino Acids and Amino Acid Standard Mixture

1. Add 100 µL of coupling reagent to the dried hydrolyzed or amino acid standard sample (*see* **Note 10**).
2. Lyophilize (*see* **Note 10**).
3. Add a further 100 µL of coupling reagent and 5 µL of phenylisothiocyanate (PITC) to the dried sample.
4. Incubate the mixture at room temperature for 5 min.
5. Lyophilize the products.
7. Add 100 µL UHQ water to the dried products (*see* **Note 11**).
8. Lyophilize (*see* **Note 11**).
9. Resuspend products in 200 µL of sample loading buffer and analyze an aliquot by RP-HPLC as described in **Subheading 3.4**.

3.4. RP-HPLC Separation of Derivatized Amino Acids

1. Connect the flow to the Supelcosil LC-18-DB HPLC column within the column heater compartment equilibrated at 45°C.
2. Wash the column with solvent B until a stable baseline is observed (*see* **Note 12**).
3. Load the amino acid analysis gradient parameters and allow the system to equilibrate at the initial conditions (*see* **Table 1** and **Note 13**).
4. Once the baseline has stabilized inject 10 µL of the sample loading buffer and run the gradient shown in **Table 1**.

Table 1
Reversed Phase-High Performance Liquid Chromatography Conditions for Separation of PTC-Derivatized Amino Acids

Time (min)	% Solvent	% Solvent	% Solvent	Flow rate (mL/min)
	A	B	C	
0	75	5	20	1.0
25	30	50	20	1.0
28	20	80	0	1.0
33	20	80	0	1.0
34	75	5	20	1.0

5. Visually examine the chromatogram obtained and ensure that there is no significant UV absorption in the region of interest (between 5 and 25 min following injection; *see* **Fig. 1**). If the chromatogram contains no significant absorbance in the region of interest proceed to **step 6.** If UV peaks are observed the column should be cleaned or a new column used and a second aliquot of sample loading buffer should be analyzed.
6. Inject sequentially six separate 10 µL aliquots of the derivatized amino acid standard mixture.
7. Examine the resulting chromatograms for peak area variation, elution time variation, peak resolution, and tailing to determine that the system is suitable for use. Peak resolution should be calculated using the equation:

$$R=2(t_2-t_1)$$

W_2+W_1 Where, t_2 and t_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline (*see* **Fig. 2**). Peak tailing should be calculated using the equation:

$$^{W}0.05$$

2f Where $W_{0.05}$ is the width of the peak at 5% height and, f is the distance from the peak front to the peak center at 5% peak height (*see* **Fig. 3**). If the system is suitable for use (*see* **Note 14**) analysis of samples may continue. If the data do not pass the set criteria, a test regime must be established to determine the reason for the failure (*see* **Note 15**).

8. Inject sequentially duplicate aliquots (10 µL) of the samples of interest.
9. Following each run sample peaks should be integrated taking into account the internal standard. The response ratio for each amino acid in each sample run is calculated by dividing the peak area for each amino acid by the peak area obtained for the internal standard (norleucine) in the same run.

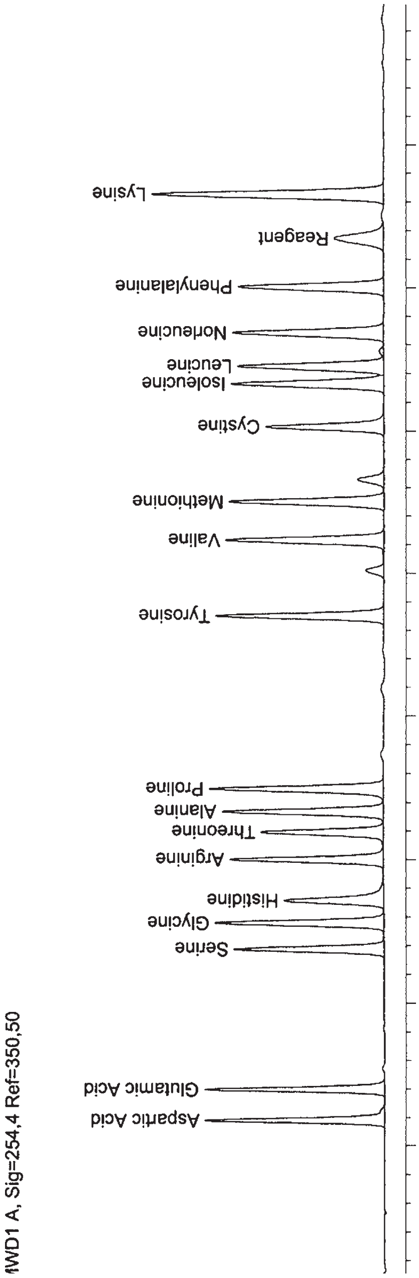

Fig. 1. UV chromatogram (254 nm) obtained from analysis of a standard mixture of derivatized amino acids.

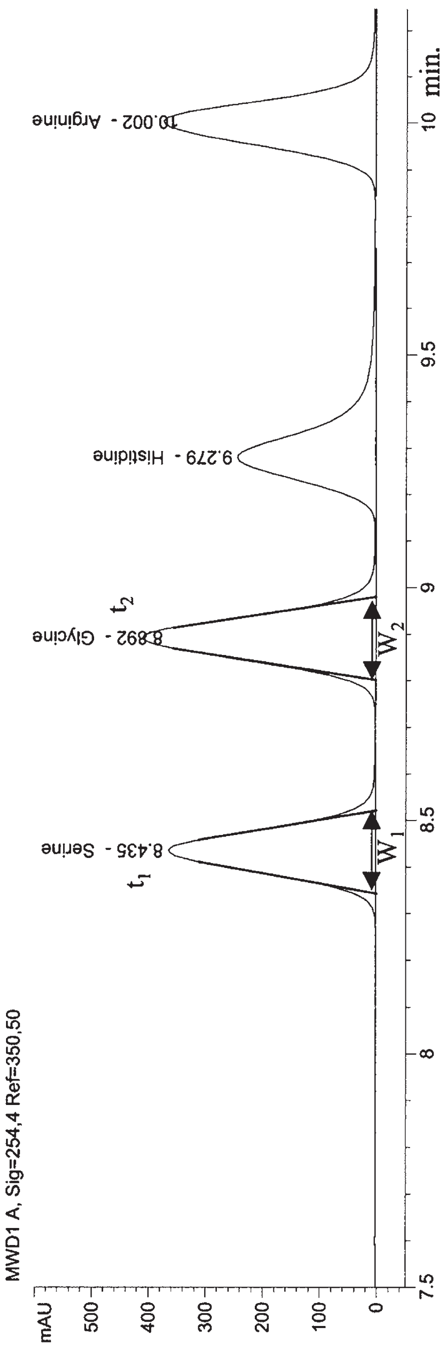
MWD1 A, Sig=254,4 Ref=350,50
mAU
500
400
300
200
100
0
7.5
8
8.5
9
9.5
10
min.
8.435 - Serine
8.892 - Glycine
9.279 - Histidine
10.002 - Arginine
t_1
t_2
W_1
W_2

Fig. 2.

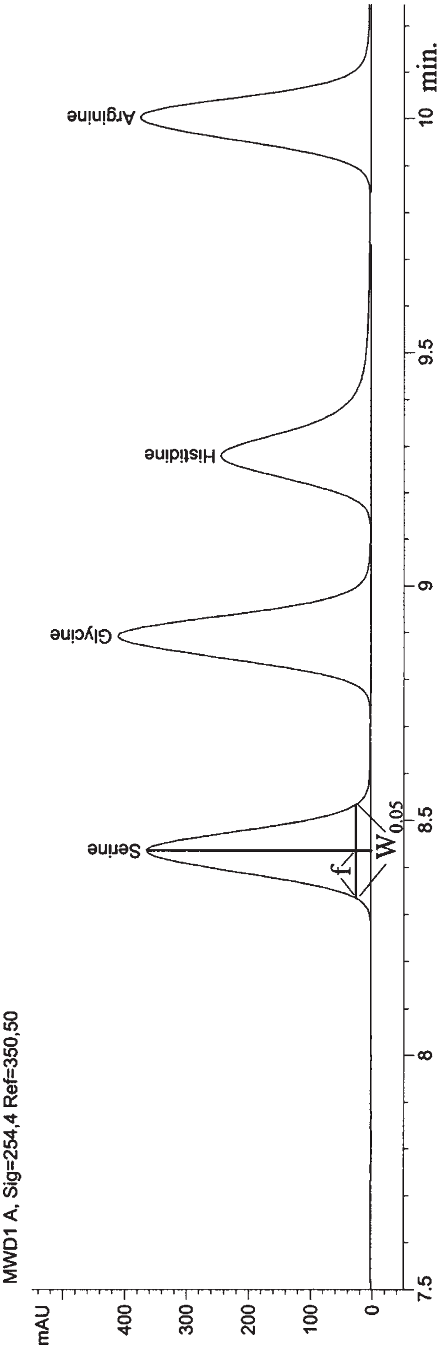
MWD1 A, Sig=254,4 Ref=350,50
mAU
400
300
200
100
0
7.5
8
8.5
9
9.5
10 min.
Serine
Glycine
Histidine
Arginine
f
$W_{0,05}$

Fig. 3.

Calculated by dividing the peak area for each amino acid by the peak area obtained for the internal standard (norleucine) in the same run.

The response ratio is in turn divided by the response ratio for the same amino acid in the amino acid standard mixture. The figure generated is multiplied by the known amount of the relative amino acid present in the standard mixture (i.e., 50 nmoles) to produce the molar amount of that amino acid in the sample. This may be summarized in the equations,

Rr = Rs/Ris (in the sample data)
Rm = Rs/Ris (in the standard mixture data)
Rc = Rr/Rm

nmoles of amino acid = Rc × 50 Where R_r is the response ratio for a particular amino acid in the sample or standard run of interest having a peak area R_s and an internal standard peak area (in the same run) R_{is} and, R_m is the response ratio for the same amino acid in the relevant standard mixture having a peak area R_s and an internal standard peak area (in the same run) R_{is}. and, R_c is the overall response ratio.

3.5. Validation Parameters (see Note 16)

The validation parameters that should be assessed during a full validation to ICH guidelines are outlined in **Subheadings 3.5.1.–3.5.9**. Samples should be prepared as outlined in **Subheadings 3.1.–3.4**. or using another suitable amino acid analysis procedure.

3.5.1. Specificity

Specificity is defined as the ability to assess unequivocally the analyte(s) in the presence of other components.

A suitable study should be designed to establish retention times and responses of known amino acids released from the study sample and the data obtained should be compared to that obtained from a standard mixture containing derivatized amino acids.

Generally, a standard aliquot of the peptide, protein, or conjugate for which the validation is being carried out is hydrolyzed and analyzed in duplicate and the data generated is compared with the data obtained from derivatized phenylthiocarbamyl (PTC)-amino acid standards.

The retention time of each of the PTC-amino acids released from the peptide, protein, or conjugate sample should normally be within ±3% of the same PTC-amino acid present in the standard mixture analyzed in the same session.

3.5.2. System Suitability Test (see **Note 17***)*

System suitability is a test designed to ascertain the effectiveness of the operating system.

System suitability can be determined in a number of ways. Generally a series of replicate injections (n = 6) of the derivatized amino acid standards is performed to assess the relative standard deviations for peak area measurements and retention times. In addition, peak tailing (T) and peak resolution (R) can be determined (*see* **Subheading 3.3**).

The retention time variance should normally be within 3% and the relative standard deviation of peak areas taken from the six sets of data should normally be less than 5%. The peak tailing factor (T) should be less than or equal to two and the resolution factor (R) should be greater than one for the amino acids of interest.

3.5.3. Linearity

Linearity is defined as the ability (within a given range) of the analytical method to obtain results that are directly proportional to the concentration of analyte(s) in the sample.

Linearity may be demonstrated on the peptide, protein, or conjugate and standard mixture of amino acids via dilution of a standard stock solution or on separate weighings out of the peptide, protein, or conjugate.

Linearity of response of the amino acid standard mixture can be demonstrated by choosing at least 5 points in duplicate in a range to cover that anticipated for the peptide, protein, or conjugate.

This study should be repeated with aliquots of the peptide, protein, or conjugate (5 points in duplicate; 0, 50, 75, 100, and 150% of the usual aliquot analyzed).

Linearity should initially be determined using a visual inspection of a plot of peak area ratio (area of amino acid signal over the area of internal standard signal) against relative amount of the peptide, protein, or conjugate taken. If a linear relationship exists the plot obtained should be statistically evaluated. For example the sum of least squares around the regression line (r^2) should be calculated.

From the final plot, the correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be determined and entered in the validation document.

The linearity data obtained should obey the equation $y = mx + C$, where C is zero within 95% confidence limits, and the sum of least squares around the regression line (r^2) is greater than 0.98.

3.5.4. Range

The range is derived from the linearity study and is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of peptide, protein, or conjugate within or at the extremes of the specified range of the analytical procedure.

Following the linearity procedure described above, the working range for the analysis would be 50–150% of the concentration of the batch used for the analysis.

If the working range required is likely to be wider, additional concentrations may be added to the linearity study to take into account the future expected concentration range.

3.5.5. Accuracy/Recovery

An assay method should provide a true (accurate) result for a test peptide, protein, or conjugate sample.

Accuracy should be established across the specified range of the analytical procedure and can often be inferred once precision, linearity, and specificity have been established.

Generally percentage recoveries for the amino acids of interest should fall within the range of 90–110% and the percentage correlation of variance (%CV; standard deviation � 100 / mean) for multiple measurements should normally be less than 10%.

3.5.6. Repeatability

Repeatability is measured by carrying out analysis (in six replicates) in one laboratory by one operator, using one instrument over a relatively short time span.

A standard aliquot of the peptide, protein or conjugate is hydrolyzed and the derivatized products are injected six times onto the RP-HPLC system. In general, the percentage correlation of variance (%CV) for the released amino acids should be less than or equal to 5%.

3.5.7. Intermediate Precision

Intermediate Precision is defined as long term variability of the measurement process, which may be determined for a method run on different days and or by different operators.

Six separate aliquots of the peptide, protein, or conjugate should be hydrolyzed, derivatized and analyzed by RP-HPLC on different days. The analyses should be carried out using different operators and different instruments in the same facility (*see* **Note 18**).

In general, the percentage correlation of variance (%CV) for the released amino acids should be less than or equal to 10%.

3.5.8. Reproducibility

Reproducibility expresses the precision between laboratories and can be assessed using an extension of the intermediate precision study described in **Subheading 3.5.7** to include analyses at different laboratories.

Ideally, the percentage correlation of variance (%CV) for the released amino acids should be less than or equal to 10%.

3.5.9. Robustness/Ruggedness

Robustness/ruggedness is defined as a review of the critical parameters in the method and the steps taken to minimise method variability. Generally experiments should be designed to assess the following criteria:

1. Stability of analytical solutions and derivatized products.
2. Influence of variations of pH in a mobile phase.
3. Influence of variations in mobile-phase composition.
4. Different columns (different lots and/or suppliers).
5. Temperature.
6. Flow rate.

For most validation studies it is impractical to carry out analyses to study all the factors previously outlined. The use of experimental matrix software can often be used to design a set of experiments to test the robustness/ruggeddness of the method being validated. Acceptance criteria are set prior to the start of the analyses and should adhere to the criteria set for linearity and percentage correlation of variance (%CV) described earlier. Unfortunately there are no actual in-depth guidelines to aid us in designing robustness/ruggeddness analyses.

3.5.10. Detection and Quantification Limit (i.e., Minimum Amount of Amino Acid Needed for Reliable Detection and Quantification)

Several approaches for determining the detection limit are possible ***(2)***. However, during a validation of amino acid analysis detection limits for each amino acid can be most easily determined based on the standard deviation of the response and the slope of the linearity plot.

The detection limit *(DL)* may be expressed as:

$$DL = {}^{3.3\alpha}S$$

Where � � the standard deviation of the response about the line of best fit S � the slope of the calibration curve The quantification limit (QL) may be determined in the same way and is expressed as:

$$QL = {}^{10\alpha}S$$

where � � the standard deviation of the response, and *S* � the slope of the calibration curve.

4. Notes

For other methods of amino acid analysis some details may differ, according to equilibration times, column lifetime, sensitivity (e.g., the amount of standard required), and so forth, but the general principles of validation outlined above apply. Ideally, the HPLC system used should meet the basic criteria specified in the liquid chromatography Pharmacopoeias ***(3–5)***.

1. It is important to remember that the main objective of validation of an analytical procedure is to demonstrate that the procedure is suitable for its intended purpose.
2. All of the analytical data collected during a validation and any calculations made should be discussed in the resulting validation document as appropriate.
3. Well-characterized reference materials, with documented purity, should be used throughout a validation study.
4. The experimental work should be designed so that the appropriate validation characteristics can be considered simultaneously to provide a sound, overall knowledge of the capabilities of the analytical procedure.
5. Analyst information, performance validation, and/or calibration certificates for the equipment used should also be recorded and stored along with the raw data. All reagents should be approved for use prior to any validation study that is to be included in a regulatory authority application and their details (supplier, catalog number, lot number and expiry dates) recorded.
6. A Hewlett-Packard HP 1100 or HP 1200 with quaternary switching pump is ideal. Reagents of the highest quality should be used during these analyses.
7. If validation is being carried out for a protein or amino acid mixture that contains unusual amino acids or amino acids that are not present in the standard mixture, the unusual amino acid should be purchased, accurately weighed, and added to the standard mixture. If the amino acid is not commercially available, the amino acid can either be synthesized, rigorously characterized, accurately weighed, and added to the standard mixture or quantitated relative to a similar amino acid.
8. Ideally prior to hydrolysis peptide, protein and conjugate samples should be salt-, amine-, and detergent-free, because these may interfere with hydrolysis or subsequent chromatography.
9. Inter-amino acid bonds differ in their susceptibility to acid hydrolysis. For example, longer acid hydrolyses are more likely to yield accurate results for hydrophobic amino acid residues, such as Valine, Isoleucine, and Phenylalanine. Other factors also have to be taken into account when utilizing amino acid analysis data. Asparagine and Glutamine are converted to their corresponding acids, Aspartic acid and Glutamic acid, and therefore cannot be directly quantified. Losses of Serine, Threonine, and to an extent Tyrosine, can be expected during acid hydrolysis through hydrolytic breakdown. More accurate estimates of Serine and Threonine can often be determined

either by assuming 10% and 5% losses, respectively, over 24 h at 110°C, or by performing a series of hydrolyses for various times and extrapolating the amounts of these amino acids detected back to zero time. Determination of Cysteine is also problematic when using standard hydrolysis conditions, due to oxidation. Tryptophan is completely destroyed during normal hydrolyses and is not detected. Also, the quantitation of Aspartic and Glutamic acid, in precolumn derivatization methods, can also be compromised by the presence of buffer salts. Proteins are routinely hydrolyzed at 110°C for 24 h. Higher recoveries of more hydrophobic amino acids from membrane proteins can be achieved using higher-tempera-ture hydrolyses for example 160°C for 6 h. This matter is discussed further in Chapter 10.

10. Addition of coupling buffer and subsequent lyophilization ensures neutralization of the hydrolysed sample and the amino acid standard mixture.
11. The addition of UHQ water to the derivatized amino acids and subsequent lyophilization ensures optimal removal of derivatizing reagents and prevents variable chromatography.
12. The column routinely needs to be flushed for 30 min with solvent B to ensure that the column and UV lamp have equilibrated.
13. Equilibration at the initial conditions for amino acid analysis normally requires 5–10 min.
14. The system can be considered suitable if the retention time variance is ±3.0% and the relative standard deviation in peak areas for the six runs is < 5%. Peak tailing (T) should be less than or equal to two and Resolution (R) should be greater than one.
15. If the system fails a suitability test, the liquid chromatography system being used including hardware (pump, UV detector, switching valves, column, etc.) and solvents should be fully examined, for instance for leaks or partial blockages. Columns have a finite lifetime. For instance the type of RP-HPLC column used in the system described in **Subheading 2.1.1**. would normally be expected to allow 150–300 sample injections before peak broadening and loss of resolution result in excessively poor performance.
16. Validation of amino acid analysis for a particular sample should only be contemplated following optimization of the method or methods to be employed. In general the optimized method, the sample formulation and, of course, amino acid sequence for each peptide, protein, and conjugate will differ. Therefore, amino acid analysis should be validated for each product rather than using a generic validated method for analysis of various products.
17. Following validation the system suitability test should ideally be performed prior to each analysis of peptide, protein, or conjugate, providing that the

data obtained passes the criteria set the system is deemed to be suitable for analysis.

18. Precision across different instruments need only be assessed if more than one instrument is to be used for analysis of the sample of interest.

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Molecular Weight Estimation for Native Proteins Using High-Performance Size-Exclusion Chromatography

G. Brent Irvine

1. Introduction

The chromatographic separation of proteins from small molecules on the basis of size was first described by Porath and Flodin, who called the process "gel filtration" ***(1)***. Moore applied a similar principle to the separation of polymers on crosslinked polystyrene gels in organic solvents, but named this "gel permeation chromatography" ***(2)***. Both terms came to be used by manufacturers of such supports for the separation of proteins, leading to some confusion. The term size-exclusion chromatography is more descriptive of the principle on which separation is based and has largely replaced the older names, although the expression "gel filtration" is still commonly used by biochemists to describe separation of proteins in aqueous mobile phases. Comprehensive reviews that cover both practical and theoretical aspects of the technique are available, with emphasis on both proteins ***(3,4)*** and peptides ***(5)***, and there is also much useful information in a technical booklet published by a leading manufacturer ***(6)***.

Conventional liquid chromatography is carried out on soft gels, with flow controlled by peristaltic pumps and run times on the order of several hours. These gels are too compressible for use in high-performance liquid chromatography (HPLC), but improvements in technology have led to the introduction of new supports with decreased particle size (5–13 µm) and improved rigidity. This technique is usually called high-performance size-exclusion chromatography (HPSEC), but is sometimes referred to as size-exclusion high-performance liquid chromatography (SE-HPLC). Columns (about 1 × 30 cm) are sold prepacked with the support, and typically have many thousands of theoretical plates. They

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can be operated at flow rates of about 1 mL/min, giving run times of about 12 min, 10–100 times faster than conventional chromatography on soft gels. A medium pressure high-performance system called fast protein liquid chromatography (FPLC) that avoids the use of stainless steel components was developed by one major supplier (Amersham Pharmacia Biotech, now GE Healthcare Life Sciences). Prepacked columns of Superose and Superdex developed for this system can also be operated on ordinary HPLC systems.

As well as being a standard chromatographic mode for desalting and purifying proteins, size-exclusion chromatography can be used for estimation of molecular weights. This is true only for ideal size-exclusion chromatography, in which the support does not interact with solute molecules (*see* **Note 1**). Because the physical basis for discrimination in size-exclusion chromatography is size, one would expect that there would be some parameter related to the dimensions and shape of the solute molecule that would determine its K_d value (*see* **Note 2**). All molecules with the same value for this parameter should have identical K_d values on a size-exclusion chromatography column. Dimensional parameters that have been suggested for such a "universal calibration" include two terms related to the hydrodynamic properties of proteins, namely Stokes radius (R_S) and viscosity radius (R_h). Current evidence appears to indicate that R_h is more closely correlated to size-exclusion chromatography behavior than is R_S *(7)*. However, even this parameter is not suitable for a universal calibration that includes globular proteins, random coils, and rods *(8)*. Hence calibration curves prepared with globular proteins as standards cannot be used for the assignment of molecular weights to proteins with different shapes, such as the rodlike protein myosin. Several mathematical models that relate size-exclusion chromatography to the geometries of protein solutes and support pores have been proposed (*see* **Note 3**). However, in practical terms, for polymers of the same shape, plots of log molecular weight against K_d have been found to give straight lines within the range $0.1 < K_d < 0.9$ (*see* **Note 4**). It has been found that the most reliable measurements of molecular weight by HPSEC are obtained under denaturing conditions, when all proteins have the same random coil structure. Disulfide bonds must be reduced, usually with dithiothreitol, in a buffer that destroys secondary and tertiary structure. Buffers containing guanidine hydrochloride *(9,10)* or sodium dodecyl sulfate (SDS) *(11)* have been used for this purpose. However, the use of denaturants has many drawbacks, which are described in **Note 5**. In any case, polyacrylamide gel electrophoresis in SDS-containing buffers is widely used for determining the molecular weights of protein subunits. This technique can also accommodate multiple samples in the same run, although each run takes longer than for HPSEC. More recently SEC has been used in combination with other more absolute methods for molecular weight determination, particularly mass spectrometry *(12)* and multiangle laser light scattering *(13)*.

The method described in this chapter is for the estimation of molecular weights of native proteins. The abilities to measure bioactivity, and to recover native protein in high yield, make this an important method, even though a number of very basic, acidic, or hydrophobic proteins will undergo non-ideal size exclusion under these conditions.

2. Materials

2.1. Apparatus

An HPLC system for isocratic elution is required. This comprises a pump, an injector, a size-exclusion column and a guard column (*see* **Note 6**), a UV-detector, and a data recorder. There are many size-exclusion columns based on surface-modified silica on the market. The results described here were obtained using a Zorbax Bioseries GF-250 column (0.94 × 25 cm), of particle size 4 µm, sold by Agilent Technologies. This column can withstand very high back pressures (up to 380 Bar or 5500 psi) and can be run at flow rates up to 2 mL/min with little loss of resolution ***(14)***. It has a molecular weight exclusion limit for globular proteins of several hundred thousand.

The exclusion limit for the related GF-450 column is about a million. Other suitable columns include TSK-GEL SW columns (Tosoh Biosep), and Superdex and Superose columns (Amersham Pharmacia Biotech).

2.2. Chemicals

1. 0.2 *M* Disodium hydrogen orthophosphate (Na_2HPO_4).
2. 0.2 *M* Sodium dihydrogen orthophosphate (NaH_2PO_4).
3. 0.2 *M* Sodium phosphate buffer, pH 7.0: Mix 610 mL of 0.2 *M* Na_2HPO_4 with 390 mL of 0.2 *M* NaH_2PO_4. Filter through a 0.22-µm filter (Millipore type GV).
4. Solutions of standard proteins: Dissolve each protein in 0.2 *M* sodium phosphate buffer, pH 7.0 at a concentration of about 0.5 mg/mL. Filter the samples through a 0.45-µm filter (Millipore type HV). Proteins suitable for use as standards are listed in **Table 1** (*see* **Note 7**).
5. Blue dextran, average molecular weight 2,000,000 (Sigma), 1 mg/mL in 0.2 *M* sodium phosphate buffer, pH 7.0. Filter through a 0.45-µm filter (Millipore type HV).
6. Glycine, 10 mg/mL in 0.2 *M* sodium phosphate buffer, pH 7.0. Filter through a 0.45-µm filter (Millipore type HV).

3. Method

1. Allow the column to equilibrate in the 0.2 *M* sodium phosphate buffer, pH 7.0, at a flow rate of 1 mL/min (*see* **Note 8**) until the absorbance at 214 nm is constant.
2. Inject a solution (20 µL) of a very large molecule, such as blue dextran (*see* **Note 9**) to determine V_0. Repeat with 20 µL of water (negative absorbance peak) or a solution of a small molecule, such as glycine, to determine V_t.

Table 1
Protein Standards

Protein	Molecular weight
Thyroglobulin	669,000
Apoferritin	443,000
β-Amylase	200,000
Immunoglobulin G	160,000
Alcohol dehydrogenase	150,000
Bovine serum albumin	66,000
Ovalbumin	42,700
β-Lactoglobulin	36,800
Carbonic anhydrase	29,000
Trypsinogen	24,000
Soybean trypsin inhibitor	20,100
Myoglobin	16,900
Ribonuclease A	13,690
Insulin	5900
Glucagon	3550

All proteins listed were obtained from Sigma, Poole, England.

3. Inject a solution (20 µL) of one of the standard proteins and determine its elution volume, V_e, from the time at which the absorbance peak is at a maximum. Repeat this procedure until all the standards have been injected. A chromatogram showing the separation of seven solutes during a single run on a Zorbax Bio-series GF-250 column is shown in **Fig. 1**. Calculate K_d (*see* **Note 2**) for each protein and plot K_d against log molecular weight. A typical plot is shown in **Fig. 2**.
4. Inject a solution (20 µL) of the protein of unknown molecular weight and measure its elution volume, V_e, from the absorbance profile. If the sample contains more than one protein, and the peaks cannot be assigned with certainty, collect fractions and assay each fraction for the relevant activity.
5. Calculate K_d for the unknown protein and use the calibration plot to obtain an estimate of its molecular weight.

4. Notes

1. Silica to which a hydrophilic phase such as a diol has been bonded still contains underivatized silanol groups. Above pH 3.0 these are largely anionic and will interact with ionic solutes, leading to non-ideal size-exclusion chromatography. Depending on the value of its isoelectric point, a protein can be cationic or anionic at pH 7.0. Proteins that are positively charged will undergo ion exchange, causing them to be retarded. Conversely, anionic proteins will experience electrostatic repulsion from the pores, referred to as "ion exclusion," and will be eluted earlier than

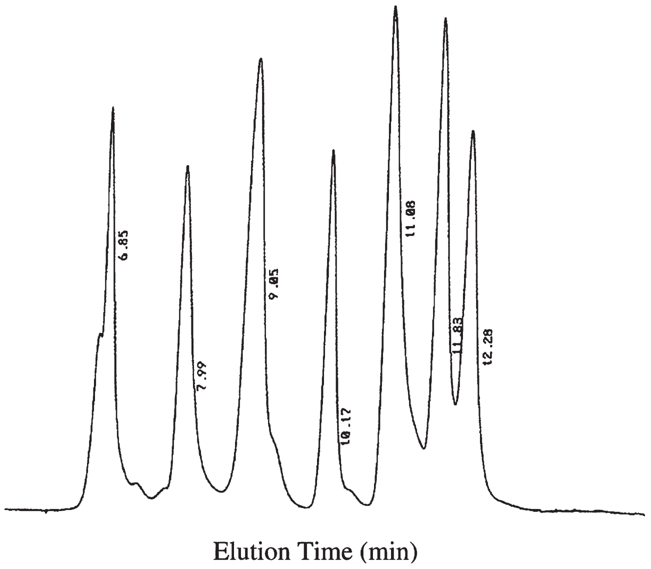

Fig. 1. Separation of a mixture of seven solutes on a Zorbax Bio-series GF-250 column. Twenty microliters of a mixture containing about 1.5 µg of each protein was injected. The solutes were, in order of elution, thyroglobulin, alcohol dehydrogenase, ovalbumin, myoglobin, insulin, glucagon, and sodium azide. The number beside each peak is the elution time in minutes. The absorbance of the highest peak, insulin, was 0.105. The equipment was a Model 501 Pump, a 441 Absorbance Detector operating at 214 nm, a 746 Data Module, all from Waters (Millipore, Milford, MA, USA) and a Rheodyne model 7125 injector (Rheodyne, Cotati, CA, USA) with 20-µL loop. The column was a Zorbax Bio-Series GF-250 with guard column (Aligent Technologies). The flow rate was 1 mL/min and the chart speed was 1 cm/min. The attenuation setting on the Data Module was 128.

expected on the basis of size alone. When size exclusion chromatography is carried out at a low pH, the opposite behavior is found, with highly cationic proteins being eluted early and anionic ones being retarded. To explain this behavior, it has been suggested that at pH 2.0 the column may have a net positive charge ***(15)***. To reduce ionic interactions it is necessary to use a mobile phase of high ionic strength. On the other hand, as ionic strength increases, this promotes the formation of hydrophobic interactions. To minimize both ionic and hydrophobic interactions, the mobile phase should have an ionic strength between 0.2 and 0.5 *M* ***(16)***.

2. The support used in size-exclusion chromatography consists of particles containing pores. The molecular size of a solute molecule determines the degree to which it can penetrate these pores. Molecules that are wholly excluded from the packing emerge from the column first, at the void volume, V_0. This represents the volume in the interstitial space (outside the

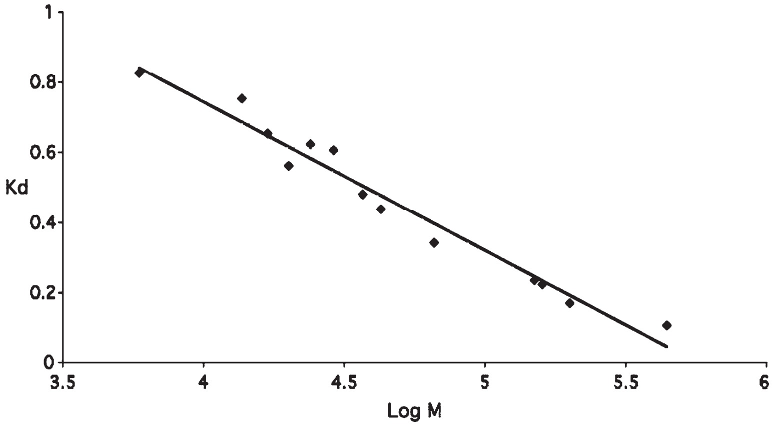

Fig. 2. Plot of K_d vs log molecular weight for those proteins listed in **Table 1** with K_d values between 0.1 and 0.9 (i.e., all except glucagon and thyroglobulin). Chromatography was carried out as described in the caption to **Fig. 1**. V_0 was determined to be 6.76 mL from the elution peak of blue dextran. Vt was determined from the elution peak of glycine and from the negative peak given by injecting water, both of which gave a value of 11.99 mL. The regression line $y = -0.4234x + 2.4378$, $r^2 = 0.9657$ was computed using all the points shown.

support particles) and is determined by chromatography of very large molecules, such as blue dextran or DNA. Molecules that can enter the pores freely have full access to an additional space, the internal pore volume, V_i. Such molecules emerge at V_t, the total volume available to the mobile phase, which can be determined from the elution volume of small molecules. Hence $V_t = V_0 + V_i$. A solute molecule that is partially restricted from the pores will emerge with elution volume, Ve, between the two extremes, V_0 and V_t. The distribution coefficient, K_d, for such a molecule represents the fraction of V_i available to it for diffusion. Hence

$$Ve = V0 + K\mathrm{d}Vi \text{ and } K\mathrm{d} = (V_e - V_0)/V_i = (V_e - V_0)/(V_t - V_0)$$

3. These models give rise to various plots that should result in a linear relationship between a function of solute radius, usually R_S, and some size-exclusion chromatography parameter, usually K_d ***(17)***. However, none has proven totally satisfactory. Recently it has been suggested that size-exclusion chromatography does not require pores at all, but rather that K_d can be calculated from a thermodynamic model for the free energy of mixing of the solute and the gel phase ***(18)***.
4. Manufacturers' catalogs often show plots of K_d against log molecular weight, M. For most globular proteins, this plot is a sigmoidal curve that is approximately linear in the middle section, where

$$Kd = a - b \log M$$

where a is the intercept on the ordinal axis, and b is the slope. From such plots one can estimate fractionation range (the working range lies in the linear portion, between about $0.9 > K_d > 0.1$) and the selectivity. This latter parameter depends on the slope of the plot and is a function of the pore size distribution. A support with average pore size distribution in a narrow band gives high selectivity (large value of b) but a restricted separation range (the larger the value of b, the lower the range of values for M). If no estimate is available for the size of the protein under investigation it is better to use a support with a broad fractionation range. A support of higher selectivity in a more restricted fractionation range can then be used later to give a more precise value for solute size.

5. Problems arising when size-exclusion chromatography is carried out under denaturing conditions include:

   a. For a particular column, the molecular weight range in which separation occurs is reduced. This is because the radius of gyration, and hence the hydrodynamic size, of a molecule increases when it changes from a sphere to a random coil. For example, the separation range of a TSK G3000SW column operating with denatured proteins is 2000–70,000, compared to 10,000–500,000 for native proteins ***(10,19)***. Of course this may actually be an advantage when working with small proteins or peptides.
   b. Proteins are broken down into their constituent subunits and polypeptide chains, so that the molecular weight of the intact protein is not obtained.
   c. Bioactivity is usually destroyed or reduced and it is not usually possible to monitor enzyme activity. This can be a serious disadvantage when trying to identify a protein in an impure preparation.
   d. The denaturants usually absorb light in the far UV range, so that monitoring the absorbance in the most sensitive region for proteins (200–220 nm) is no longer possible.
   e. Manufacturers often advise that once a column has been exposed to a mobile phase containing denaturants it should be dedicated to applications using that mobile phase, as the properties of the column may be irreversibly altered. In addition, the denaturant, especially if it is SDS, may be difficult to remove completely.
   f. Because these mobile phases have high viscosities, flow rates may have to be reduced to avoid high back pressures.
   g. High concentrations of salts, especially those containing halide ions, can adversely affect pumps and stainless steel.

6. Most manufacturers sell guard columns appropriate for use with their size-exclusion columns. To protect the expensive size-exclusion column it is strongly recommended that a guard column be used.
7. Even in the presence of high ionic strength buffers several proteins show nonideal behavior and are thus unsuitable as standards. For example, the basic proteins cytochrome c (pI ≅10) and lysozyme (pI ≅11) have K_d values > 1.0, under the conditions described for **Fig. 1**, because ion-exchange interactions are not totally suppressed. On the other hand, the very acidic protein pepsin (pI ≅1) emerges earlier than expected on the basis of size, because of ion exclusion. One should be aware that such behavior might also occur when interpreting results for proteins of unknown pI.
8. Columns are often stored in ethanol–water mixtures or in 0.02% sodium azide to prevent bacterial growth. Caution: Sodium azide is believed to be a mutagen. It should be handled with care (see suppliers' safety advice) and measures taken to avoid contact with solutions. It can also lead to explosions when disposed of via lead pipes. Solutions should be collected in waste bottles.

   When changing mobile phase some manufacturers recommend that the flow rate should not be greater than half the maximum flow rate.
9. Although V_0 is most commonly measured using blue dextran, Himmel and Squire suggested that it is not a suitable marker for the TSK G3000SW column, owing to tailing under nondenaturing conditions, and measured V_0 using glutamic dehydrogenase from bovine liver (Sigma Type II; mol. wt 998,000) ***(20)***. Calf thymus DNA is also a commonly used marker for V_0.

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Detection of Disulfide-Linked Peptides by HPLC

Alastair Aitken and Michèle Learmonth

1. Introduction

Classical techniques for determining disulfide bond patterns usually require the fragmentation of proteins into peptides under low pH conditions to prevent disulfide exchange. Pepsin or cyanogen bromide are particularly useful (*see* Chapters 101 and 94 respectively).

Diagonal techniques to identify disulphide-linked peptides were developed by Brown and Hartley (*see* Chapter 113). A modern micromethod employing reverse-phase high-performance liquid chromatography (HPLC) is described here.

2. Materials

1. 1 *M* Dithiothreitol (DTT, good quality, e.g., Calbiochem).
2. 100 m*M* Tris-HCl, pH 8.5.
3. 4-Vinylpyridine.
4. 95% Ethanol.
5. Isopropanol.
6. 1 M triethylamine-acetic acid, pH 10.0.
7. Tri-n-butyl-phosphine (1% in isopropanol).
8. HPLC system such as Dionex UltiMate Micro HPLC systems for LC and LC/MS.
9. Reverse phase HPLC columns such as Vydac C_4, C_8, C_{18} microbore (1.0 mm I.D. columns) for the purification of low picomole amounts (< 5 pmol) of peptides. (*see* **Note 1**).

3. Method (*see* refs. 1 and 2)

1. Alkylate the protein (1–10 mg in 20–50 mL of buffer) without reduction to prevent possible disulfide exchange by dissolving in 100 m*M* Tris-HCl, pH 8.5, and adding 1 µL of 4-vinylpyridine (*see* **Note 2**).

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2. Incubate for 1 h at room temperature and desalt by HPLC or precipitate with 95% ice-cold ethanol followed by bench centrifugation.
3. The pellet obtained after the latter treatment may be difficult to redissolve and may require addition of 10-fold concentrated acid (HCl, formic, or acetic acid) before digestion at low pH. It may be sufficient to resuspend the pellet with acid using a sonic bath if necessary, then commence the digestion. Vortex-mix the suspension during the initial period until the solution clarifies.
4. Fragment the protein under conditions of low pH (*see* **Note 3**) and subject the peptides from half the digest to reverse-phase HPLC. Vydac C_4, C_8, or C_{18} columns give particularly good resolution depending on the size range of fragments produced.

Typical separation conditions are;- column equilibrated with 0.1% (v/v) aqueous trifluoro acetic acid (TFA), elution with an acetonitrile–0.1% TFA gradient. A combination of different cleavages, both chemical and enzymatic, may be required if peptide fragments of interest remain large after one digestion method.

5. To the other half of the digest (dried and resuspended in 10 µL of isopropanol) add 5 µL of 1 *M* triethylamine-acetic acid pH 10.0; 5 µL of tri-*n*-butyl-phosphine (1% in isopropanol) and 5 µL of 4-vinylpyridine. Incubate for 30 min at 37 °C, and dry *in vacuo,* resuspending in 30 µL of isopropanol twice. This procedure cleaves the disulfides and modifies the resultant –SH groups.
6. Run the reduced and alkylated sample on the same column, under identical conditions on reverse-phase HPLC. Cysteine-linked peptides are identified by the differences between elution of peaks from reduced and unreduced samples.
7. Collection of the alkylated peptides (which can be identified by rechromatography on reverse-phase HPLC with detection at 254 nm) and a combination of sequence analysis and mass spectrometry *(see* **Note 1** and Chapter 112) will allow disulfide assignments to be made.

4. Notes

1. If the HPLC separation is combined with mass spectrometric characterization, the level of TFA required to produce sharp peaks and good resolution of peptide (approx 0.1% v/v) results in almost or complete suppression of signal. This does not permit true on-line HPLC-MS as the concentration of TFA in the eluted peptide must first be drastically reduced. However, the "low TFA," 218MS54, reverse-phase HPLC columns from Vydac (300 Å pore size) are available in C_4 and two forms of C_{18} chemistries. They are also supplied in 1 mm diameter columns that are ideal for low levels of sample eluted in minimal volume. We have used as little as 0.005% TFA without major loss of resolution and have observed minimal signal loss. There may be a difference in selectivity compared to "classical" reverse-phase columns; for example, we have observed phosphopeptides eluting approx. 1% acetonitrile later than their unphosphorylated counterparts (the

opposite to that conventionally seen). This is not a problem, but it is something of which one should be aware, and could be turned to advantage.

2. The iodoacetic acid used must be colorless. A yellow color indicates the presence of iodine; this will rapidly oxidize thiol groups, preventing alkylation and may also modify tyrosine residues. It is possible to recrystallize from hexane. Reductive alkylation may also be carried out using iodo-[^{14}C]-acetic acid or iodoacetamide (*see* Chapter 82). The radiolabelled material should be diluted to the desired specific activity before use with carrier iodoacetic acid or iodoacetamide to ensure an excess of this reagent over total thiol groups.
3. Fragmentation of proteins into peptides under low pH conditions to prevent disulfide exchange is important. Pepsin, Glu-C, or cyanogen bromide are particularly useful (*see* Chapters 99 and 94 respectively). Typical conditions for pepsin are 25 °C for 1–2 h at pH 2.0–3.0 (10 m*M* HCl, 5% acetic or formic acid) with an enzyme:substrate ratio of about 1:50. Endoproteinase Glu-C has a pH optimum at 4 as well as an optimum at pH 8.0. Digestion at the acid pH (typical conditions are 37 °C overnight in ammonium acetate at pH 4.0 with an enzyme/substrate ratio of about 1:50) will also help minimize disulfide exchange. CNBr digestion in guanidinium 6 *M* HCl/ 0.1–0.2 *M* HCl may be more suitable acid medium due to the inherent redox potential of formic acid which is the most commonly used protein solvent. When analyzing proteins that contain multiple disulfide bonds it may be appropriate to carry out an initial chemical cleavage (CNBr is particularly useful), followed by a suitable proteolytic digestion. The initial acid chemical treatment will cause sufficient denaturation and unfolding as well as peptide bond cleavage to assist the complete digestion by the protease. If a protein has two adjacent cysteine residues this peptide bond will not be readily cleaved by specific endopeptidases. For example, this problem was overcome during mass spectrometric analysis of the disulfide bonds in insulin by using a combination of an acid proteinase (pepsin) and carboxypeptidase A as well as Edman degradation ***(3)***.

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Detection of Disulfide-Linked Peptides by Mass Spectrometry

Alastair Aitken and Michèle Learmonth

1. Introduction

Mass spectrometry is playing a rapidly increasing role in protein chemistry and sequencing and is particularly useful in determining sites of co- and post-translational modification ***(1,2)***, and application in locating disulfide bonds is no exception. This technique can of course readily analyze peptide mixtures; therefore it is not always necessary to isolate the constituent peptides. However, a cleanup step to remove interfering compounds such as salt and detergent may be necessary. Thus can be achieved using matrices such as 10-µm porous resins slurry-packed into columns 0.25 mm diameter. Polypeptides can be separated on stepwise gradients of 5–75% acetonitrile in 0.1% formic or acetic acid ***(3)***. On-line electrospray mass spectrometry (ES-MS) coupled to capillary electrophoresis or high-performance liquid chromatography (HPLC) has proved particularly valuable in the identification of modified peptides. If HPLC separation on conventional columns is attempted on-line with mass spectrometry, the level of trifluoroacetic acid (TFA) (0.1%) required to produce sharp peaks and good resolution of peptides results in almost or complete suppression of signal. In this case it is recommended to use the "low TFA," 218MS54, reverse-phase HPLC columns from Vydac (300 Å pore size) which can be used with as little as 0.005% TFA without major loss of resolution and minimal signal loss (*see* further details in Chapter 111).

Sequence information is readily obtained using triple quadrupole tandem mass spectrometry after collision-induced dissocation ***(4)***. Ion trap mass spectrometry technology (called LCQ) is now well established which also permits sequence information to be readily obtained. Not only can MS-MS analysis be

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carried out, but owing to the high efficiency of each stage, further fragmentation of selected ions may be carried out to MS.” The charge state of peptide ions is readily determined by a “zoom-scan” technique that resolves the isotopic envelopes of multiply charged peptide ions. The instrument still allows accurate molecular mass determination to 100,000 Da at 0.01% mass accuracy. The recent development of the Orbitrap mass spectrometer ***(5)*** allows very high resolution and sensitivity.

Selective detection of modified peptides is possible on ES-MS. For example, phosphopeptides can be identified from the production of phosphate-specific fragment ions of 63 Da (PO) and 79 Da (PO) by collision-induced dissociation during negative ion HPLC–ES-MS. This technique of selective detection of posttranslational modifications through collision-induced formation of low-mass fragment ions that serve as characteristic and selective markers for the modification of interest has been extended to identify other groups such as glycosylation, sulfation and acylation ***(6)***.

2. Materials

Materials for proteolytic and chemical cleavage of proteins are described in Chapters 94–99.

3. Method

3.1. Detection of Disulfide-Linked Peptides by Mass Spectrometry

1. Peptides generated by any suitable proteolytic or chemical method that minimizes disulfide exchange (i.e., acid pH, *see* **Note 1**). Partial acid hydrolysis, although nonspecific, has been successfully used in a number of instances. The peptides are then analyzed by a variety of mass spectrometry techniques ***(7)***. The use of thiol and related compounds should be avoided for obvious reasons. Despite this, it is possible that disulfide bonds will be partially reduced during the analysis and peaks corresponding to the individual components of the disulfide-linked peptides will be observed. Control samples with the above reagents are essential to avoid misleading results (*see* **Note 2**).
2. The peptide mixture is incubated with reducing agents, such as mercaptoethanol and dithiothreitol (DTT), and reanalyzed as before. Peptides that were disulfide linked disappear from the spectrum and reappear at the appropriate positions for the individual components. For example, in the positive ion mode the mass (M) of disulfide-linked peptides (of individual masses A and B) will be detected as the pseudomolecular ion at $(M+H)^+$, and after reduction this will be replaced by two additional peaks for each disulfide bond in the polypeptide at masses $(A+H)^+$ and $(B+H)^+$. Remember that $A + B = M + 2$, as reduction of the disulfide bond will be accompanied by a consistent increase in mass due to the conversion of cystine to two cysteine residues, that is, $-S\text{-}S \rightarrow -SH + HS\text{-}$ and peptides containing an intramolecular disulfide bond will appear at 2 amu higher. Such peptides, if they

are in the reduced state can normally be readily reoxidized to form intramolecular disulfide bonds by bubbling a stream of air through a solution of the peptide for a few minutes (*see* **Note 3**).

3. Computer programs are readily available on the Internet that are supplied with the mass spectrometer software package and will predict the cleavage position of any particular proteinase or chemical reagent. Simple knowledge of the mass of the fragment will, in most instances, give unequivocal answers as to which segments of the polypeptide chain are disulfide linked. If necessary, one cycle of Edman degradation can be carried out on the peptide mixture and the mass spectroometry analysis repeated. The shift in mass(es) that correlates with loss of specific residues will confirm the assignment.

4. Notes

1. Fragmentation of proteins into peptides under low pH conditions is important (e.g., with pepsin, Glu-C, or cyanogen bromide; *see* Chapter 99 and 94 respectively) to prevent disulfide exchange. Analysis can also be carried out by electrospray mass spectrometers (ES-MS), which will give an accurate molecular mass up to 80–100,000 Da (and in favorable cases up to 150,000 Da). The increased mass of 2 Da for each disulfide bond will be too small to obtain an accurate estimate for a polypeptide of mass Ca 10,000 (accuracy obtainable is >0.01%). There has been a recent marked increase in resolution obtained with both electrospray mass spectrometers and laser desorption time-of-flight mass spectrometers that could now permit a meaningful analysis. On the other hand, oxidation with performic acid will cause a mass increase of 48 Da for each cysteine and 49 Da for each half-cystine residue. Note that Met and Trp will also be oxidized. Met sulfoxide, the result of incomplete oxidation and that is often found after gel electrophoresis, has a mass identical to that of Phe. Met sulfone is identical in mass to Tyr. Orbitrap mass spectrometers have ppm accuracy that is well within the necessary range of resolution even for medium to large proteins ***(8)***.
2. Mass analysis by ES-MS ***(3,9)*** and matrix-assisted laser desorption mass spectrometry with time-of-flight detection (MALDI-TOF) ***(10)*** is affected, seriously in some cases, by the presence of particular salts, buffers, and detergents. In some cases, using nonionic saccharide detergents such as *n*-dodecyl-β-D-glucopyranoside, improvements in signal-to-noise ratios of peptides and proteins were observed ***(10)***. The effect on ES-MS sensitivity of different buffer salts, detergents, and tolerance to acid type may vary widely with the instrument and particularly with the ionization source. Critical micelle concentration is not a good predictor of how well a surfactant will perform ***(9)***.
3. The difference of 2 Da may allow satisfactory estimation of the number of intramolecular disulfide bonds by mass spectrometry. If necessary a larger

mass difference may be generated by oxidation with performic acid ***(11)***, and *see* Chapter 83). This will cause a mass increase of 48 Da for each cysteine and 49 Da for each half-cystine residue. (Remember that Met and Trp will also be oxidized.) ***(12)*** or by exopeptidase digestion from the N- and C-termini. This essentially a subtractive technique (one looks at the mass of the remaining fragment after each cycle). For example, when a phosphoserine residue is encountered, a loss of 167.1 Da is observed in place of 87.1 for a serine residue. This technique therefore avoids one of the major problems of analyzing posttranslational modifications. Although the majority of modifications are stable during the Edman chemistry, *O*- or *S*-linked esters, for example (which are very numerous), may be lost by β-elimination (e.g., *O*-phosphate) during the cleavage step to form the anilinothioazolidone or undergo *O*- (or *S*- in the case of palmitoylated cysteine) to *N*-acyl shifts which block further Edman degradation. Exopeptidase digestion may be difficult and the rate of release of amino acid may vary greatly.

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Diagonal Electrophoresis for Detecting Disulfide Bridges

Alastair Aitken and Michèle Learmonth

1. Introduction

Methods for identifying disulfide bridges have routinely employed "diagonal" procedures using two-dimensional paper or thin-layer electrophoresis. This essentially utilizes the difference in electrophoretic mobility of peptides containing either cysteine or cystine in a disulfide link, before and after oxidation with performic acid. It was first described by Brown and Hartley ***(1)***. Peptides unaltered by the performic acid oxidation have the same mobility in both dimensions and, therefore, lie on a diagonal. After oxidation, peptides that contain cysteine or were previously covalently linked produce one or two spots off the diagonal, respectively. This method has also been adapted for HPLC methodology and is discussed in Chapter 111.

First the protein has to be fragmented into suitable peptides containing individual cysteine residues. It is preferable to carry out cleavages at low pH to prevent possible disulfide bond exchange. In this respect, pepsin (active at pH 3.0) and cyanogen bromide (CNBr) are particularly useful reagents. Proteases with active-site thiols should be avoided (e.g., papain, bromelain).

Before the advent of HPLC, paper electrophoresis was the most commonly used method for peptide separation ***(2)***. Laboratories with a history of involvement with protein characterization are likely to have retained the equipment, but it is no longer commercially available. Although it is possible to make a simple electrophoresis tank in house ***(3)***, thin-layer electrophoresis equipment is still commercially available, and it is advisable that this be used, owing to the safety implications.

For visualization of the peptides, ninhydrin is the classical amino group stain. However, if amino acid analysis or sequencing is to be carried out, fluorescamine is the reagent of choice.

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2. Materials

2.1. Equipment

1. Electrophoresis tank.
2. Flat bed electrophoresis apparatus (e.g., Hunter thin-layer peptide-mapping apparatus (CBS Scientific, Del Mar, CA,. and VWR International Ltd, UK).
3. Whatman (Maidstone, UK) 3MM and Whatman No. 1 paper.
4. Cellulose TLC plates (Camlab [Cambridge, UK]

2.2. Reagents

1. Electrophoresis buffers (*see* **Note 1**): Commonly used volatile buffers are:

   pH 2.1 acetic acid/formic acid/water 15/5/80, v/v/v
   pH 3.5 pyridine/acetic acid/water 5/50/945 v/v/v
   pH 6.5 pyridine/acetic acid/water 25/1/225, v/v/v

2. Nonmiscible organic solvents: toluene, for use with pH 6.5 buffer: white spirit for use with pH 2.1 and 3.5 buffers.
3. Formic acid. **Care!**
4. 30% w/v Hydrogen peroxide. **Care!**
5. Fluorescamine: 1 mg/100 mL in acetone.
6. Marker dyes: 2% orange G, 1% acid fuschin, and 1% xylene cyanol dissolved in appropriate electrophoresis buffer.
7. 1% (v/v) Triethylamine in acetone.

3. Methods

3.1. First-Dimension Electrophoresis

3.1.1. Paper Electrophoresis

1. Dissolve the peptide digest from 0.1–0.3 µmol protein in 20–25 µL electrophoresis buffer.
2. Place the electrophoresis sheet on a clean glass sheet (use Whatman No. 1 for analytical work, Whatman 3MM for preparative work). Support the origin at which the sample is to be applied on glass rods. Where paper is used, multiple samples can be run side by side. Individual strips can then be cut out for running in the second dimension.
3. Apply the sample slowly without allowing to dry (covering an area of about 2 × 1 cm) perpendicular to the direction intended for electrophoresis. NB: for pH 6.5 buffer, apply near the center of the sheet, for acidic buffers, apply nearer the anode end.
4. Apply a small volume of marker dyes (2% orange G, 1% acid fuschin, 1% xylene cyanol, in electrophoresis buffer) on the origin and additionally in a position that will not overlap the peptides after the second dimension.
5. Once the sample is applied, wet the sheet with electrophoresis buffer slowly and uniformly on either side of the origin so that the sample concentrates in a thin line. Remove excess buffer from the rest of the sheet with blotting paper.
6. Place the wet sheet in the electrophoresis tank previously set up with electrophoresis buffer covering bottom electrode. An immiscible organic solvent (toluene

where pH 6.5 buffer is used, white spirit for acidic buffers) used to fill the tank to the top. Upper trough filled with electrophoresis buffer. **Care!**

7. Electrophorese at 3 kV for about 1 h, with cooling if necessary. The progress of the electrophoresis can be monitored by the movement of the marker dyes (*see* **Note 1**).
8. Dry sheet in a well-ventilated place overnight, at room temperature, secured from a glass rod with Bulldog clips.

3.1.2. Thin-Layer Electrophoresis

1. Dissolve the peptide digest from 0.1–0.3 µmol protein in at least 10 µL electrophoresis buffer.
2. Mark the sample and dye origins on the cellulose side of a TLC plate with a cross using an extrasoft blunt-ended pencil, or on the reverse side with a permanent marker.
3. Spot the sample on the origin. This can be done using a micropipet fitted with a disposable capillary tip. To keep the spot small, apply 0.5–1 µL spots and dry between applications.
4. Apply 0.5 µL marker dye to the dye origin.
5. Set up electrophoresis apparatus with electrophoresis buffer in both buffer tanks. Prepare electrophoresis wicks from Whatman 3MM paper, wet with buffer, and place in buffer tanks.
6. Prepare a blotter from a double sheet of Whatman 3MM, with holes cut at the positions of the sample and marker origins. Wet with buffer and place over TLC plate. Ensure concentration at the origins by pressing lightly around the holes.
7. Place TLC plate in apparatus.
8. Electrophorese at 1.5 kV for about 30–60 min.
9. Dry plate in a well-ventilated place at room temperature, overnight.

3.2. Performic Acid Oxidation (see Note 2)

1. Prepare performic acid by mixing 19 mL formic acid with 1 mL 30% (w/v) hydrogen peroxide. The reaction is spontaneous. **Care!**
2. Place the dry electrophoresis sheet/plate in a container where it can be supported without touching the sides.
3. Place the performic acid in a shallow dish inside the container. Close the container and leave to oxidize for 2–3 h. (Note the marker dyes change from blue to green.)
4. Dry sheet thoroughly at room temperature overnight.

3.3. Second Dimension Electrophoresis

3.3.1. Paper Electrophoresis

1. To prepare for the second dimension, individual strips from the first dimension can be machine zigzag stitched onto a second sheet. The overlap of the second sheet should be carefully excised with a razor blade/scalpel.
2. Wet the sheet with electrophoresis buffer, applying the buffer along both sides of the sample, thus concentrating the peptides in a straight line.
3. Repeat electrophoresis, at right angles to the original direction.
4. Thoroughly dry sheet, as before.

3.3.2. Thin-Layer Electrophoresis

1. Wet TLC plate with electrophoresis buffer using two sheets of prewetted Whatman 3 MM paper on either side of sample line.
2. Repeat electrophoresis at right angles to the original direction.
3. Thoroughly dry plate as before.

3.4. Visualization

Peptide spots can be seen after reaction with fluorescamine.

1. The reaction should be carried out under alkaline conditions. The sheet or plate should be dipped in a solution of triethylamine (1% [v/v]) in acetone. This should be carried out at least twice if the electrophoresis buffer employed was acidic. Dry the sheet well.
2. Dip the sheet in a solution of fluorescamine in acetone (1 mg/100 mL).
3. Allow most of the acetone to evaporate.
4. View the map under a UV lamp at 300–365 nm (**Care:** Goggles must be worn). Peptides and amino-containing compounds fluoresce. Encircle all fluorescent spots with a soft pencil.
5. Interpretation of diagonal maps: **Fig. 1** shows that peptides unaltered by the performic acid treatment have the same mobility in both dimensions and therefore lie

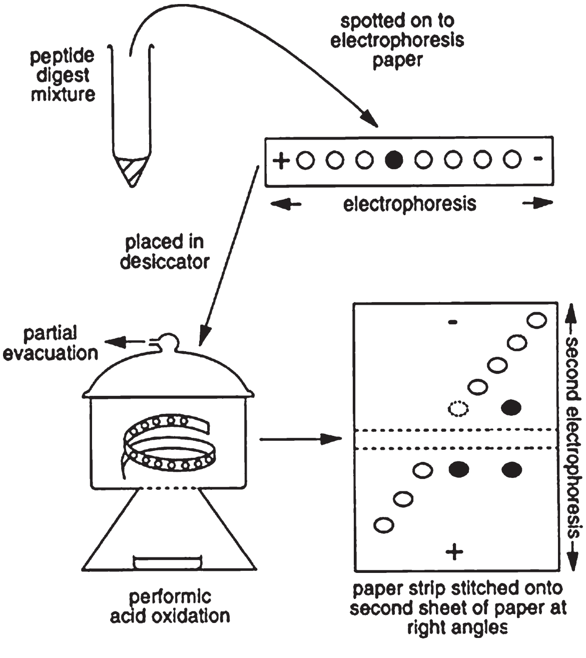
peptide digest mixture
spotted on to electrophoresis paper
+ ○ ○ ○ ● ○ ○ ○ ○ -
electrophoresis
placed in desiccator
partial evacuation
performic acid oxidation
-
+
second electrophoresis
paper strip stitched onto second sheet of paper at right angles

Fig. 1. Diagonal electrophoresis for identification and purification of peptides containing cysteine or disulfide bonds. This figure shows that peptides unaltered by the performic acid treatment (open circles) have the same mobility in both dimensions and therefore lie on a diagonal. Peptides that contain cysteine or were previously covalently linked (closed circles) produce one or two spots respectively, that lie off the diagonal after oxidation.

on a diagonal. Peptides that contain cysteine or were previously covalently linked produce one or two spots, respectively, that lie off the diagonal after oxidation.

3.5. Elution

Peptides may be eluted from the paper using, for example, $0.1 M$ NH_3 or 25% acetic acid. Peptides may be extracted from TLC plates by scraping off the spot into an Eppendorf tube containing elution buffer. This should be vortexed for 5 min and then centrifuged for 5 min. The cellulose can then be re-extracted once or twice with the same buffer to ensure optimal recovery of peptide.

4. Notes

1. The buffer of choice for the initial analysis is pH 6.5. However, if the cysteine residues have already been blocked with iodoacetate (*see* Chapter 82), the pH 3.5 buffer is very useful, since peptides containing these residues lie slightly off the diagonal, being slightly more acidic in the second dimension after the performic acid oxidation.
2. The movement of the marker dyes will enable progress of the electrophoresis to be followed to ensure that the samples do not run off the end of the paper.
3. It is important to exclude halide ions rigorously, since these are readily oxidized to halogens, which will react with histidine, tyrosine, and phenylalanine residues in the protein.

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Estimation of Disulfide Bonds Using Ellman's Reagent

Alastair Aitken and Michèle Learmonth

1. Introduction

Ellman's reagent 5,5' -dithiobis(2-nitrobenzoic acid) (DTNB) was first introduced in 1959 for the estimation of free thiol groups ***(1)***. The procedure is based on the reaction of the thiol with DTNB to give the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) which is quantified by the absorbance of the anion (TNB^{2-}) at 412 nm.

The reagent has been widely used for the quantitation of thiols in peptides and proteins. It has also been used to assay disulfides present after blocking any free thiols (e.g., by carboxymethylation) and reducing the disulfides prior to reaction with the reagent ***(2,3)***.

It is also commonly used to check the efficiency of conjugation ***(4)*** of sulfhydryl-containing peptides to carrier proteins (such as maleimide activated keyhole limpet haemocyanin, KLH) in the production of antibodies and to ensure that cysteine labeled sythetic peptides are coupled to affinity resins.

2. Materials

1. Reaction buffer: 0.1 *M* phosphate buffer, pH 8.0.
2. Denaturing buffer: 6 *M* guanidinium chloride, 0.1 *M* Na_2HPO_4, pH 8.0 (*see* **Note 1**).
3. Ellman's solution: 10 m*M* (4 mg/mL) DTNB (Pierce, Chester, UK) in 0.1 *M* phosphate buffer, pH 8.0 (*see* **Note 2**).
4. Dithiothreitol (DTT) (Boerhinger or Calbiochem) solution: 200 m*M* in distilled water.

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3. Methods

3.1. Analysis of Free Thiols

1. It may be necessary to expose thiol groups, which may be buried in the interior of the protein. The sample may therefore be dissolved in reaction buffer or denaturing buffer. A solution of known concentration should be prepared with a reference mixture without protein. Sufficient protein should be used to ensure at least one thiol per protein molecule can be detected; in practice, at least 2 nmol of protein (in 100 µL) are usually required.
2. Sample and reference cuvets containing 3 mL of the reaction buffer or denaturing buffer should be prepared and should be read at 412 nm. The absorbance should be adjusted to zero (*A*buffer).
3. Add 100 µL of buffer to the reference cuvet.
4. Add 100 µL of Ellman's solution to the sample cuvet. Record the absorbance (A_{DTNB}).
5. Add 100 µL of protein solution to the reference cuvet.
6. Finally, add 100 µL protein solution to the sample cuvet, and after thorough mixing, record the absorbance until there is no further increase. This may take a few minutes. Record the final reading (A_{final}).
7. The concentration of thiols present may be calculated from the molar absorbance of the TNB anion. (*See* **Note 3**.)

$$\Delta A_{412} = E_{412}TNB^{2-}[RSH] \qquad (1)$$

$$\text{Where } \Delta A_{412} = A_{final} - (3.1/3.2)\ (A\text{DTNB} - A\text{buffer})\ -1_{M}-1$$

$$\text{and } E_{412}\ TNB^{2-} = 1.415 \times 10^{4}\ \text{cm}.$$

If using denaturing buffer, use the value $E_{412}\ TNB^{2-} = 1.415 \times 10^{4}\ \text{cm}^{-1}M^{-1}$.

3.2. Analysis of Disulfide Thiols

1. Sample should be carboxymethylated (*see* Chapter 82) or pyridethylated (*see* Chapter 85) without prior reduction. This will derivatize any free thiols in the sample, but will leave intact any disulfide bonds.
2. The sample (at least 2 nmol of protein in 100 µL, is usually required) should be dissolved in 6 *M* guanidinium HCl, 0.1 *M* Tris-HCl, pH 8.0 or denaturing buffer, under a nitrogen atmosphere.
3. Add freshly prepared DTT solution to give a final concentration of 10–100 m*M*. Carry out reduction for 1–2 h at room temperature.
4. Remove sample from excess DTT by dialysis for a few hours each time, with two changes of a few hundred mL of the reaction buffer or denaturing buffer (*see* **Subheading 3.1**). Alternatively, gel filtration into the same buffer may be carried out.
5. Analysis of newly exposed disulfide thiols can thus be carried out as described in **Subheading 3.1.**

4. Notes

1. It is not recommended to use urea in place of guanidinium HCl, since this can readily degrade to form cyanates, which will react with thiol groups.
2. Unless newly purchased, it is usually recommended to recrystallize DTNB from aqueous ethanol.
3. Standard protocols for use of Ellman's reagent often give E_{412} TNB^{2-} = 1.36×10^4 cm $^{-1}M^{-1}$. A more recent examination of the chemistry of the reagent indicates that these are more suitable values ***(5)***, and these have been used in this chapter.

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Quantitation of Cysteine Residues and Disulfide Bonds by Electrophoresis

Alastair Aitken and Michèle Learmonth

1. Introduction

Amino acid analysis quantifies the molar ratios of amino acids per mole of protein. This generally gives a nonintegral result, yet clearly there are integral numbers of the amino acids in each protein. A method was developed by Creighton ***(1)*** to count integral numbers of amino acid residues, and it is particularly useful for the determination of cysteine residues. Sulfhydryl and disulfide groups are of great structural, functional, and biological importance in protein molecules. For example, the Cys sulfhydryl is essential for the catalytic activity of some enzymes (e.g., thiol proteases) and the interconversion of Cys SH to Cystine S—S is directly involved in the activity of protein disulfide isomerase ***(2)***. The conformation of many proteins is stabilized by the presence of disulfide bonds ***(3)***, and the formation of disulfide bonds is an important posttranslational modification of secretory proteins ***(4)***.

Creighton's method exploits the charge differences introduced by specific chemical modifications of cysteine. A similar method was first used in the study of immunoglobulins by Feinstein in 1966 ***(5)***. Cys residues may be reacted with iodoacetic acid, which introduces acidic carboxymethyl ($—O_2CCH_2S—$) groups, or with iodoacetamide, which introduces the neutral carboxyamidomethyl ($H_2NCOCH_2S—$) groups. The reaction with either reagent is essentially irreversible, thereby producing a stable product for analysis. Using a varying ratio of iodoacetamide/iodoacetate, these acidic and neutral agents will compete for the available cysteines, and a spectrum of fully modified protein molecules having 0,1,2, … *n* acidic carboxymethyl residues per molecule is produced (where *n* is the number of cysteine residues in the protein). These species will

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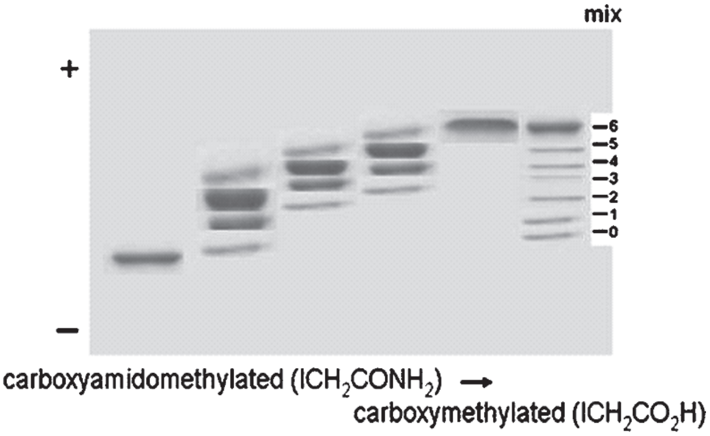
mix
+
−
−6
−5
−4
−3
−2
−1
−0
carboxyamidomethylated (ICH_2CONH_2) →
carboxymethylated (ICH_2CO_2H)

Fig. 1. Idealised electrophoretic pattern in an analysis of a protein with six cysteine residues, run with the low-pH system. Lanes 1–5 (left to right) contain (respectively) samples reacted with neutral iodoacetamide; 1:1; 1:3; 1:9 ratios of neutral to acidic reagent; acidic iodoacetate. The right hand lane contains a mixture of equal portions of the samples in lanes 1–5.

have, correspondingly, n, $n - 1$, $n - 2$, … 0 neutral carboxyamidomethyl groups. These species may then be separated by electrophoresis, isoelectric focusing, or by a combination of both *(1,6,7)*. The theoretical analysis of a protein with 6 cysteines is shown in **Fig. 1**.

Creighton used a low-pH discontinuous system *(1)*. Takahasi and Hirose recommend a high-pH system *(6)*, whereas Stan-Lotter and Bragg used the Laemmli electrophoresis system followed by isoelectric focusing *(7)*. It may therefore be necessary to carry out preliminary experiments to find the best separation conditions for the protein under analysis. The commonly used methods are given below.

In order to ensure that all thiol groups are chemically equivalent, the reactions must be carried out in denaturing (in the presence of urea) and reducing (in the presence of dithiothreitol, DTT) conditions. The electrophoretic separation must also be carried out with the unfolded protein (i.e., in the presence of urea) in order that the modification has the same effect irrespective of where it is in the polypeptide chain.

The original method has been modified into a two-stage process to allow for the quantification of both sulfhydryl groups and disulfide bonds (*see* **Notes 1** and **2**) *(6,8)*. The principle of the method has also been adapted to counting the numbers of lysine residues after progressive modification of the ε-amino groups with succinic anhydride, which converts this basic group to a carboxylic acid-containing moiety.

2. Materials

2.1. Reaction Solutions

1. 1 *M* Tris-HCl, pH 8.0.
2. 0.1 *M* EDTA, pH 7.0.
3. 1 *M* DTT (good-quality, e.g., Calbiochem, Nottingham, UK).
4. 8 *M* Urea (BDH [Poole, UK], Aristar-grade, *see* **Note 2**).
5. Solution A: 0.25 *M* iodoacetamide, 0.25 *M* Tris-HCl, pH 8.0.
6. Solution B: 0.25 *M* iodoacetic acid, prepared in 0.25 *M* Tris-HCl, pH readjusted to 8.0 with 1 *M* KOH.

2.2. Solutions for Electrophoretic Analysis in the Low-pH System (pH 4.0)(9)

1. 30% Acrylamide solution containing 30 g acrylamide, 0.8 g *bis*-acrylamide **(extreme caution: work in fume hood)**, made up to 100 mL with distilled water.
2. 10% Acrylamide solution containing 10 g acrylamide **(extreme caution: work in fume hood)** and 0.25 g *bis*-acrylamide made up to 100 mL with distilled water.
3. Low-pH buffer (eight times concentrated stock) for separating gel; 12.8 mL glacial acetic acid, 1 mL *N,N,N N* -tetramethylethylenediamine (TEMED), 1 *M* KOH (approx 35 mL) to pH 4.0, made up to 100 mL with distilled water.
4. Low pH buffer (8 times concentrated) for stacking gel; 4.3 mL glacial acetic acid, 0.46 mL TEMED, 1 *M* KOH to pH 5.0, to 100 mL with distilled water.
5. 4 mg riboflavin/100 mL water.
6. Low-pH buffer for electrode buffer; dissolve 14.2 g β-alanine in 800 mL water then adjust to pH 4.0 with acetic acid. Make up to a final volume of 1 L with distilled water.
7. Tracking dye solution (five times concentrated); 20 mg methyl green, 5 mL water, and 5 g glycerol.

2.3. Gel Solution Recipes for Low-pH Electrophoresis (pH 4.0) (see Note 3)

1. 30 mL Separating gel (10% acrylamide, photopolymerized with riboflavin) is made up as follows: 10 mL 30% acrylamide stock, 4 mL pH 4.0 buffer stock, 3 mL riboflavin stock, 14.7 g urea, water (approx 2.5 mL) to 30 mL. Degas on a water vacuum pump (to removeoxygen which inhibits polymerization).
2. 8 mL Stacking gel (2.5% acrylamide, photopolymerized with riboflavin) is made up with: 2 mL 10% acrylamide stock, 1 mL pH 5.0 buffer stock, 1 mL riboflavin stock, 3.9 g urea, water (approx 1.2 mL) to 8 mL. Degas.

2.4. Electrophoresis Buffers for High-pH Separation (pH 8.9)

1. 30% Acrylamide solution containing 30 g acrylamide, 0.8 g *bis*-acrylamide **(extreme caution: work in fume hood)**, made up to 100 mL with distilled water.
2. 10% Acrylamide solution containing 10 g acrylamide **(extreme caution: work in fume hood)** and 0.25 g *bis*-acrylamide made up to 100 mL with distilled water.

3. High-pH buffer (four times concentrated stock) for separating gel; 18.2 g Tris base (in ~40 mL water), 0.23 mL TEMED, 1 *M* HCl to pH 8.9, made up to ~100 mL with distilled water.
4. High-pH buffer (four times concentrated) for stacking gel; 5.7 g Tris base (in ~40 mL water), 0.46 mL TEMED, 1 *M* H_3PO_4 to pH 6.9, made up to 100 mL with distilled water.
5. 4 mg Riboflavin in 100 mL water
6. 10% Ammonium persulfate solution (consisting of 0.1 g ammonium persulfate in 1 mL water).
7. High-pH buffer for electrode buffer; 3 g Tris base, 14.4 g glycine, distilled water to 1 L.
8. Tracking dye solution (five times concentrated): 1 mL 0.1% Bromophenol blue, 4 mL water and 5g glycerol.

2.5. Gel Solution Recipes for High-pH Electrophoresis (see Note 3)

1. 30 mL Separating gel (7.5% acrylamide, polymerized with ammonium persulfate) is made up with: 7.5 mL 30% acrylamide stock, 7.5 mL pH 8.9 buffer stock, 0.2 mL 10% ammonium persulfate (add immediately before casting), 14.7 g urea, water (approx 4.5 mL) to 30 mL. Degas.
2. 8 mL Stacking gel (2.5% acrylamide, photopolymerized with riboflavin) is made up with: 2 mL 10% acrylamide stock, 1 mL pH 6.9 buffer stock, 1 mL riboflavin stock solution, 3.9 g urea, water (approx 1.2 mL) to 8 mL. Degas.

3. Methods

3.1. Reduction and Denaturation

1. To a 0.2-mg aliquot of lyophilized protein add 10 µL of each of the solutions containing 1 *M* Tris-HCl, pH 8.0, 0.1 *M* EDTA, and 1 *M* DTT (*see* **Note 4**).
2. Add 1 mL of the 8 *M* urea solution (*see* **Note 2**).
3. Mix and incubate at 37°C for at least 30 min.

3.2. Reaction

1. Freshly prepare the following solutions using solutions A and B listed in **Subheading 2.**
    a. Mix 50 µL of solution A with 50 µL solution B (to give solution C).
    b. Mix 50 µL of solution A with 150 µL solution B (to give solution D).
    c. Mix 50 µL of solution A with 450 µL of solution B (to give solution E).
2. Label six Eppendorf tubes 1–6.
3. Add 10 µL of solutions A, B, C, D, and E to tubes 1–5. Reserve tube 6.
4. Add 40 µL of denatured, reduced protein solution prepared as in **Subheading 3.1.** to each of tubes 1–5.
5. Gently mix each tube and leave at room temperature for 15 min. Thereafter, store on ice.
6. After the 15 min incubation period, place 10 µL aliquots from each of tubes 1–5 into tube 6. Mix.

The samples are now ready for analysis (*see* **Note 5**).

3.3. Electrophoretic Analysis

1. 50 µL aliquots of each sample, Labeled 1–6, mixed with 12 µL of appropriate tracking dye solution, are loaded onto successive lanes of a polymerized high- or low-pH gel, set up in a suitable slab gel electrophoresis apparatus.
2. Low-pH buffer system: Electrophoresis is carried out toward the negative electrode, using a current of 5–20 mA for each gel, overnight at 8°C.
3. High-pH buffer system ***(10)***; Electrophoresis is carried out toward the positive electrode at 10–20 mA per gel (or 100–180 V) for 3–4 h.
4. Electrophoresis is stopped when the tracking dye reaches bottom of the gel.
5. Proteins are visualized using conventional stains e.g., Coomassie blue (Pierce, Chester, UK), silver staining (*see* Chapter 48).

4. Notes

1. The method of Takahashi and Hirose ***(6)*** can be used to categorize the half-cystines in a native protein as:
   a. Disulfide bonded;
   b. Reactive sulfhydryls; and
   c. Nonreactive sulfhydryls. In the first step, the protein sulfhydryls are alkylated with iodoacetic acid in the presence and absence of 8 *M* urea. In the second step, the disulfide bonded sulfhydryls are fully reduced and reacted with iodoacetamide. The method described above is then used to give a ladder of half-cystines so that the number of introduced carboxymethyl groups can be quantified.
2. Urea is an unstable compound; it degrades to give cyanates which may react with protein amino and thiol groups. For this reason, the highest grade of urea should always be used, and solutions should be prepared immediately before use.
3. Electrophoresis in gels containing higher or lower percent acrylamide may have to be employed depending on the molecular weight of the particular protein being studied.
4. Where protein is already in solution, it is important to note that the pH should be adjusted to around 8.0, and the DTT and urea concentrations should be made at least 10 m*M* and 8 *M,* respectively.
5. Other ratios of iodoacetic acid to iodoacetamide may need to be used if more than about eight cysteine residues are expected, since a sufficiently intense band corresponding to every component in the complete range of charged species may not be visible. A greater ratio of iodoacetic acid should be used if the more acidic species are too faint (and vice versa).

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N-Terminal Sequencing of N-Terminally Modified Proteins

Roza Maria Kamp and Hisashi Hirano

1. Introduction

A small amount of proteins separated by one-dimensional polyacrylamide gel electrophoresis (PAGE) or two-dimensional electrophoresis (2D-PAGE) and transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane by electroblotting can be sequenced directly by a gas-phase protein sequencer ***(1)***. This blotting/sequencing technique has come to be widely used in protein *de novo* sequence analysis. However, even if proteins are successfully separated by PAGE and then transferred onto a PVDF membrane, N-terminal amino acid sequences of the proteins with a blocking group at the N-terminus cannot be determined by Edman degradation in the sequencer. Many proteins are N-terminally blocked ***(2,3)***. Brown and Roberts ***(4)*** found over 50% of soluble mammalian proteins to be N-terminally blocked. Thus, a simple and rapid technique for obtaining N-terminal sequence information on blocked proteins should be developed. Most techniques proposed so far for this purpose were applicable in order to obtain only the internal sequence of proteins.

Acetyl modification is the most prevalent in the blocking groups identified so far ***(5),*** and the formyl and pyroglutamyl groups are also frequently detected (*see* **Note 1**). In this chapter, techniques for deblocking and N-terminal sequencing of proteins with an acetyl, formyl, or pyroglutamyl group at the N-terminus, which are electroblotted from a polyacrylamide gel onto a PVDF membrane ***(8)***, are described (*see* **Note 2**). Techniques for PAGE and protein electroblotting of proteins are presented elsewhere in this volume (*see also* **9,10**) (*see* **Note 3**).

On the other hand, recently, the alternative techniques using mass spectrometry have been rapidly developed, which allows the direct analysis of the N-terminally blocked peptides. The following three methods in combination with mass

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spectrometry are available. (I) Sequencing of N-terminally blocked peptide by mass spectrometry after protease digestion of proteins (*see* **Note 3**): This technique will be described for acetylated and mirystoylated proteins in this chapter ***(5)***. (II) Enrichment of N-terminal modified peptides using DITC followed by sequencing of separated peptides using mass spectrometry: The blocked N-terminal peptides can be isolated by using diisothiothyanate (DITC)-glass, which reacts only with unmodified NH_2-groups. Blocking groups, e.g. acetylated N-terminal groups do not react with DITC and can be easily and selectively isolated from all other peptides ***(6)***. Futhermore, the modified peptides can be analyzed by using mass spectrometry. (III) Specific sorting of N-terminal blocked peptides using chromatography (COFRADIC) and sequencing by mass spectrometry: Geavaert et al. described a new method, called COFRADIC, for isolation of modified N-terminal peptides. The peptides are separated using fractional diagonal chromatography. The proteins are cleaved by using endopeptidases, e.g. trypsin, and separated by using reversed phase chromatography. In the next step the internal and unblocked N-terminal peptides are treated by using 2,4,6-trinitrobenzene sulfonic acid (TNBS) and separated again by using reversed phase chromatography. The TNBS-modified peptides show strong hydrophobic shift and can be isolated from the unaltered N-terminal blocked peptides, which do not react with TNBS. The N-terminally modified peptides, e.g. acetylated peptides can be identified using mass spectrometry ***(7)***.

2. Materials

2.1. Deblocking of N-Formylated Proteins

1. Hydrochloric acid (0.1 *M*).
2. Acetonitrile (Sequencing grade).

2.2. Deblocking of Proteins with N-Terminal Pyroglutamic Acid

1. Acetonitrile (Sequencing grade).
2. Polyvinylpyrrolidone (PVP)-40: 0.5% w/v in acetic acid (100 m*M*).
3. Phosphate buffer (0.1 *M*, pH 8.0) containing 5mM dithiothreitol (DTT) and 10 m*M* ethylenediaminetetraacetic acid (EDTA).
4. Pyroglutamyl peptidase (Takara Shuzo, Japan).

2.3. Deblocking of N-Acetylated Proteins

2.3.1. Deblocking with Trifluoroacetic Acid (TFA)

1. Trifluoroacetic acid (TFA) (Sequencing grade).
2. Nitrogen Gas.

2.3.2. Deblocking with TFA in Methanol

1. TFA (Sequencing grade).
2. Methanol (Analytical grade).

2.3.3. Deblocking with Acylamino Acid Releasing Enzyme

1. Acetonitrile (Sequencing grade).
2. PVP-40 (0.5% w/v) in acetic acid (100 m*M*).
3. Trypsin (Promega, USA, modified by reductive methylation).
4. Ammonium bicarbonate buffer (0.1 *M*, pH 8.0).
5. Pyridine (50% v/v).
6. Phenylisothiocyanate (Sequencing grade).
7. Formic acid.
8. Hydrogen peroxide (30% w/v).
9. Phosphate buffer (0.2 *M*, pH 7.2).
10. DTT (1 m*M*).
11. Acylamino acid-releasing enzyme (AARE) (Takara Shuzo).
12. Nitrogen gas.

2.4. Deblocking of Proteins with N-Acyl Groups by Pyrococcus Aminopeptidase

1. Ethylmorpholine buffer (50 m*M*, pH 8.0) containing $CoCl_2$ (0.1%, v/v).
2. *Pyrococcus furiosus* aminopeptidase (Takara Shuzo).

2.5. Sequencing of the N-terminally blocked peptides by mass spectrometry

1. Trypsin (Promega, Wisconsin, USA).
2. TFA (Sequencing grade).
3. Acetonitrile (sequencing grade)
4. Nanocapillary reversed phase column C18, 150 mm length, 0.75 µm diameter (LC Pacing, California, USA).
5. Trap column for CapLC System (Waters Corp., Massachussets, USA).
6. Electrospray ionization-quadrupole/time-of-flight mass spectrometry (ESI-Q/TOF MS)(Micromass, Manchester, UK).

3. Methods

3.1. Deblocking and Sequencing of N-Formylated Proteins

1. Separate the N-terminally blocked protein (<100 pmol) by SDS-PAGE or 2D-PAGE and electroblot it onto a PVDF membrane.
2. Cut out the portion of the PVDF membrane carrying the protein band or spot.
3. Wet the membrane with a small amount of acetonitrile and soak it in 200–500 µL 0.6 M HCl at 25°C for 24 h.
4. Wash the PVDF membrane adequately with deionized water.
5. Dry the membrane in vacuo and apply it to a gas-phase sequencer.

3.2. Deblocking and Sequencing of Proteins with N-Terminal Pyroglutamic Acid

1. Separate the N-terminally blocked protein (<100 pmol) by SDS-PAGE or 2D-PAGE and electroblot it on a PVDF membrane.

2. Cut out the portion of the PVDF membrane carrying the protein band or spot.
3. Wet the membrane with a small amount of acetonitrile and soak it in 200 µL 0.5% (w/v) PVP-40 in 100 m*M* acetic acid at 37°C for 30 min to block the unbound protein-binding sites on the membrane.
4. Wash the membrane with 5 mL of deionized water at least 10 times.
5. Soak the membrane in 100 µL 0.1 *M* phosphate buffer, pH 8.0, containing 5 m*M* DTT and 10 m*M* EDTA.
6. Add pyroglutamyl peptidase (5 µg, enzyme:substrate,1:1–1:10 w/w).
7. Incubate the reaction solution at 30°C for 24 h.
8. Wash the membrane with deionized water.
9. Dry the membrane *in vacuo* and apply it to a gas-phase sequencer (*see* **Note 4**)

3.3. Deblocking and Sequencing of N-Acetylated Proteins

3.3.1. Deblocking with TFA and Sequencing

1. Separate the N-terminally blocked protein (<200 pmol) by SDS-PAGE or 2D-PAGE and electroblot it on a PVDF membrane.
2. Cut out the portion of the PVDF membrane carrying the protein band or spot and place the membrane in an Eppendorf tube.
3. Place the Eppendorf tube in a vial (2.6 × 6 cm) and then add 100–300 µL TFA.
4. Purge the vial with nitrogen gas for 30 s, seal with a stopper, and incubate at 60°C for 30 min (*see* **Note 5**).
5. Dry the membrane in vacuo and apply to a gas-phase sequencer (*see* **Notes 6** and **7**).

3.3.2. Deblocking with TFA in Methanol

1. Incubate the N-terminally blocked protein (<100 pmol) on glass fiber filters or PVDF membranes with TFA in methanol (1:1 v/v) at 47°C for 2–3 d *(8)*.
2. Dry the sample and apply it to a gas-phase sequencer.

3.3.3. Deblocking with Acylamino Acid Releasing Enzyme and Sequencing

1. Separate the N-terminally blocked protein (<100 pmol) by SDS-PAGE or 2D-PAGE and electroblot it onto a PVDF membrane.
2. Cut out the portion of the PVDF membrane carrying the protein band or spot.
3. Wet the membrane with a small amount of acetonitrile and pretreat the membrane with 200 µL 0.5% (w/v) PVP-40 in 100 mM acetic acid at 37°C for 30 min.
4. After thorough washing with deionized water, digest the protein on the PVDF membrane with 5–10 µg trypsin (enzyme:substrate, 1:1–1:10, w/w) in 100 µL 0.1 M ammonium bicarbonate buffer, pH 8.0, containing 10% (v/v) acetonitrile, at 37°C for 24 h with shaking (*see* **Notes 8** and **9**).
5. Pool the digestion buffer containing tryptic peptides in an Eppendorf tube.
6. Wash the membrane by vortexing with 100 µL deionized water and the washing solution with the digestion buffer.
7. Evaporate the digestion mixture to dryness in vacuo and add 100 µL 50% (v/v) pyridine and 10 µL phenylisothiocyanate to react with the free, but not blocked N-terminal amino acids of tryptic peptides (*see* **Note 10**).

8. Flush with nitrogen gas, vortex the reaction solution and centrifuge at 3000 g for 1 min.
9. Discard the resultant supernatant containing reaction byproducts and excess reagent. Repeat this washing procedure three times and evaporate the sample to dryness in vacuo.
10. Prepare performic acid solution by mixing 9 mL formic acid and 1 mL 30% hydrogen peroxide and keep the mixture at room temperature for 1 h. Add 100 µL performic acid to the dried sample and, after mixing, place the tube on ice (1 h) to convert the N-terminal phenylthiocarbamyl groups of the peptide to phenylcarbamyl groups by oxidation.
11. Evaporate the sample solution to dryness in vacuo, resuspend in deionized water and again dry in vacuo.
12. Resuspend the sample in 100 µL 0.2 *M* phosphate buffer, pH 7.2, containing 1 m*M* DTT.
13. Dissolve 50 mU AARE in 50 µL of the same buffer and add to the mixture. Incubate at 37°C for 12 h to remove the N-acetylated amino acid.
14. Apply the sample solution to a polybrene-coated glass fiber filter mounted into the upper glass block of the reaction chamber of a gas-phase sequencer (*see* **Notes 11–13**).

3.4. Deblocking and Sequencing of Proteins with N-Acyl Groups by Pyrococcus Aminopeptidase

An aminopeptidase from the archaeon *Pyrococcus furiosus* can be applied to the deblocking, followed by internal sequence analysis of the N-terminally blocked proteins ***(6, 11–13)***. This enzyme can cleave all peptide bonds of protein sequentially from the N-terminus, except the peptide bond at the N-terminal side of proline. Therefore, the N-terminally blocked protein can be truncated from the N-terminus to one residue before proline after *Pyrococcus* aminopeptidase digestion. It is possible to sequence the generated polypeptide with a gas-phase sequencer (*see* **Note 14**).

1. Cut out the portion of the PVDF membrane carrying the protein (<100 pmol) band or spot (*see* **Note 15**).
2. Wash the membrane with 5 mL deionized water.
3. Immerse the membrane in 1 mL 70% (v/v) acetic acid and sonicate for 1/2 h.
4. Remove the acetic acid solution and evaporate the solution to dryness.
5. Resuspend the sample in 50 µL 50 m*M* ethylmorpholine buffer, pH 8.0, containing 0.1% (w/v) $CoCl_2$.
6. Add the *Pyrococcus* aminopeptidase solution. Incubate the sample solution at 50°C for 4 h for small peptides to 2 days for large proteins (*see* **Note 16**). The optimized enzyme:substrate molar ratio is from 1:100 for small peptides to 1:1 for large proteins.
7. Apply the sample solution to a gas-phase sequencer (*see* **Note 17**).

3.5. Sequencing of N-terminally Blocked Peptides by Mass Spectrometry

Proteins were separated by SDS-PAGE or 2D-PAGE and cleaved using trypsin (**Note 17**). The tryptic peptides were separated using nanocapillary HPLC and measured using mass spectrometry such as ESI-Q/TOF MS (*see* **Fig. 1**).

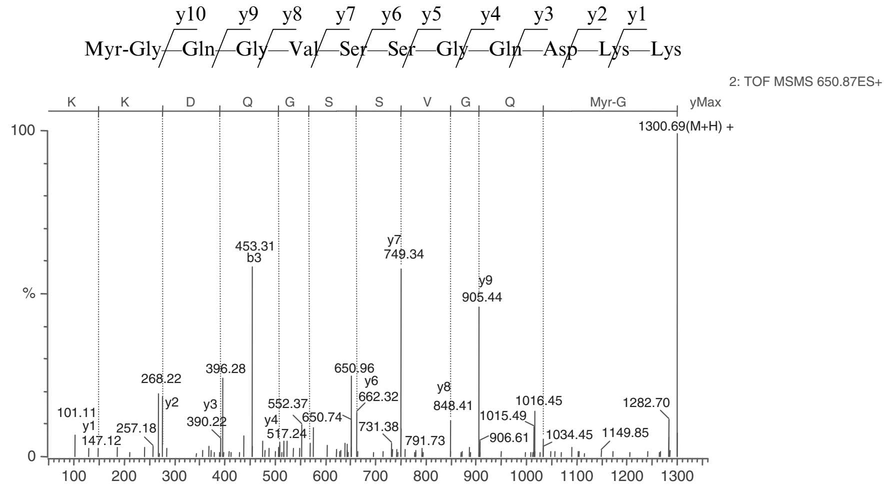
3.6. Enzymatic Digestion

1. Carefully excise the protein band from the 1D- or protein spot from 2D-gel using a scalpel.
2. Cut the gel in small 1 mm pieces and transfer in the Eppendorf tube. All used plastic or metal ware have to be washed using 100% acetonitrile for 5 min. and rinsed with deionized water before use.
3. Cover the gel pieces with 200 µL of 60%(v/v) acetonitrile and incubate at 37°C for 10 min. Remove and discard the solution from the tube.
4. Repeat the step 3 one or more times, depending of the colour intensity of stained gels.
5. Remove the solution and add 100% acetonitrile until the gel turned to opaque white.
6. Remove the acetonitrile.
7. Dry the gel pieces using lyophilization (Speed Vac Concentrator), about 15–30 min.
8. Prepare the enzyme solution (stock solution 1mg/ml trypsin in 50mM NH_4HCO_3 pH 8,0, for the digestion dilute direct before use to the concentration 12.5 ng/µl in 50m*M* NH_4HCO_3 pH 8,0).
9. Add 25 µL of the freshly prepared trypsin solution to the dryed gel. The protein has to be cover with the enzyme solution. When the 25 µL of enzyme solution are not enough add additionally 25µL.
10. Incubate at 37°C until the gel is rehydrated with the trypsin solution. If the solution is not absorbed completely by the gel, remove the excess from the tube.
11. Incubate overnight at 37°C in an stainless steel block or incubator oven. A shorter digestion about 5 h may be sufficient, but causes lower sequence coverage.
12. After the cleavage remove trypsin solution and transfer the gel pieces to the new clean Eppendorf tube.
13. Add 25 µL of 60% acetonitrile for the extraction of peptides. Shake the tube for 10 min. Repeat this procedure for 2–3 times to extract all peptides. The supernatants collect in the new tube.
14. Concentrate the sample in the Speed Vac Concentrator and store in the refrigerator at 4°C.

3.7. Separation of N-terminally Blocked Peptides using LC-MS/MS

1. Use nanocapillary reversed phase HPLC for separation of “in gel” digested peptides. Use reversed phase C18 column, buffer A 0,1% (v/v) TFA and buffer B 0,01% TFA in acetonitrile.
2. Equilibrate the column for 10 min with 0.1% TFA.
3. Inject the sample (25 nL) onto the column.

A Proteasome RPT2 N-terminal peptide Myrystoylation



B Proteasome RPT3 N-terminal peptide Acetylation

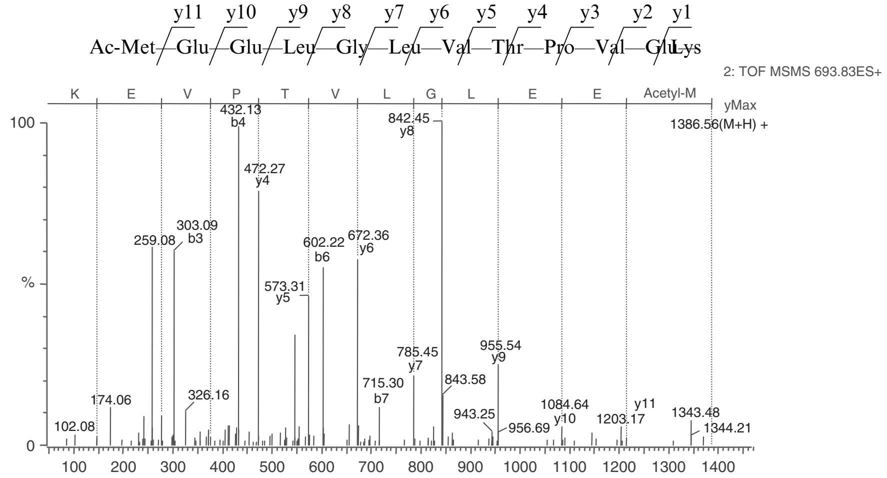


Fig. 1. ESI-MS spectrum of a) N-terminal mirystoylation of tryptic peptide from proteasome subunit RPT2 from *Saccharomyces cerevisiae*, b) N-terminal acetylation of tryptic peptide from proteasome RPT3 subunit from *Saccharomyces cerevisiae*. 4 µg of 19S proteasome was separated by SDS-PAGE, excised from the gel, digested with trypsin and 25 µl were injected in the ESI-MS.

4. Run the linear gradient 5–55% buffer B in 20 min.flow rate 500 nl/min.
5. Perform MS/MS analysis using e.g. ESI-Q/TOF MS (Micromass, Manchester, UK).

Techniques for MS analysis are presented elsewhere.

4. Notes

1. If the N-terminal peptide can be purified from a protein, the N-terminal blocking group and the sequence of the peptide may be determined by mass spectrometry.
2. Deblocking of proteins electroblotted on a PVDF membrane has several advantages:
   a. Proteins purified by PAGE can be efficiently deblocked and sequenced.
   b. Chemical and enzymatic deblocking can be easily performed without desalting, since after electroblotting on a PVDF membrane, the proteins become almost completely salt free.
   c. Proteins can be deblocked at picomole levels.
   d. Sequential deblocking as described in **Note 19** is possible.
3. Proteins are often posttranslationally modified and N-terminal blockage is a well-known posttranslational modification. Proteins are N-terminally blocked not only in vivo but also in vitro. It is possible to prevent artificial *in vitro* blocking generated during protein extraction, PAGE, and Western blotting. The use of highly pure reagents, addition of thioglycolic acid as a free-radical scavenger during the extraction, electrophoresis and electroblotting buffers, or pre-electrophoresis for removing the free-radicals from the gel may help to prevent in vitro blocking ***(14,15)***. However, if proteins are blocked in vivo, a chemical or enzymatic deblocking procedure such as that described in this chapter is required to determine the N-terminal sequence.
3. The blocked peptide can be more efficiently identified by MS, if the digests containing the N-terminally blocked peptide are roughly separated by HPLC.
4. Miyatake et al. ***(16)*** treated the protein-blotted PVDF membrane with anhydrous hydrazine vapor at 20°C for 8 h to deblock proteins with a pyroglutamic acid at the N-terminus. The N-terminal pyroglutamic acid of the protein was converted to Glu–hydrazide which then could undergo Edman degradation. But this often causes partial modification of asparagine and glutamine to their hydrazides and the conversion of arginine residue to ornithine. Milder hydrazinolysis at −5°C for 8 h may be useful to deblock the N-formylated residue ***(16)***.
5. Wellner et al. ***(17)*** indicated that an N–O acyl shift may be involved in the removal of acetyl group of N-terminal acetylserine and acetylthreonine. The advantage of this method is that deblocking is easy and rapid, although overall sequencing yields obtained by this procedure are usually low (5%).
6. When the membrane is exposed to TFA vapor for longer than 2 h, yields of the PTH-amino acids increase, but at the same time, so do those of the reaction byproducts, which prevent identification of PTH-amino acids. Reaction byproducts may be generated primarily through cleavage of polypeptide with TFA ***(18)***.
7. Proteins with either acetylserine or acetylthreonine can be deblocked by TFA treatment but not proteins with other acetylamino acids. About 36

and 4% of acetylated proteins carry acetylserine and acetylthreonine, respectively, as N-terminal amino acids. Treatment with TFA vapor should thus deblock about 40% of acetylated proteins and the remaining may be deblocked with AARE.

8. Pyroglutamated proteins with relatively high molecular mass such as hen egg riboflavin-binding protein (34 kDa) and *Geotrichum candidum* lipase (59 kDa) can be deblocked on a PVDF membrane by direct treatment with pyroglutamyl peptidase. AARE, in contrast, does not directly digest proteins of high molecular mass. As described by Tsunasawa et al. ***(19)***, AARE specifically cleaves the X-Y bond of RCO-X-Y type peptides shorter than 40 residues (R: alkyl group, X/Y: L-amino acid). Thus, prior to AARE digestion, proteins on the membrane should be digested with a protease such as trypsin. Short peptides produced are subsequently released from the membrane. In this protease digestion, 10% acetonitrile is included in the digestion buffer to reduce hydrophobic interaction between peptide and PVDF membrane ***(20)***. In this organic solvent, peptides are more efficiently eluted from the membrane. The acetylated amino acid of the N-terminal peptide extracted is selectively removed by digestion with AARE.
9. If the N-terminal peptide obtained by protease digestion has more than 10 residues, its extraction from the PVDF membrane would be difficult. In such a case, a second digestion with another protease should be performed on the same membrane.
10. Krishna et al. ***(21)*** reported an alternative method in which after fragmentation, peptides with a free amino group at the N-terminus are succinylated and the N-terminal blocked peptide is then deblocked with AARE.
11. If butyl chloride (reagent S3 in ABI protein sequencers) is delivered for 30 s prior to Edman degradation in the gas-phase sequencer to recover the N-terminal blocked amino acid released by AARE, the blocking group and N-terminal amino acid can be identified by mass spectrometry.
12. In the sequencing of cytochrome c, an N-acetylated protein, overall initial yields from successive steps including electrophoresis, electroblotting, deblocking, and sequencing ranged from 23–25%. The efficiency of deblocking and sequencing described here depends primarily on that of tryptic digestion and subsequent peptide elution from the PVDF membrane. Shaking the digestion solution containing the PVDF membrane during tryptic digestion should facilitate elution of the tryptic peptides.
13. The N-terminal myristoyl group of the blocked proteins can be removed when, instead of AARE, peptide N-fatty acylase (Wako Pure Chemicals, Japan) is used as described in **Subheading 3.3**.
14. The *Pyrococcus* aminopeptidase removes the acetylated amino acid slowly, and after cleavage of the first blocked amino acid, the speed of protein degradation rapidly increases. This makes impossible to remove only the N-terminal

blocking group with the *Pyrococcus* aminopeptidase. Therefore, the blocked N-terminal region is truncated and the internal sequence from a residue before proline can be determined by Edman degradation. Since the direct on-membrane digestion of proteins with the *Pyrococcus* aminopeptidase is not easy, it is prerequisite to elute proteins from the membranes prior to the digestion.

15. It is recommended to use the Immobilon-P membrane or teflon membrane (GoreTex). These membranes allow more efficient elution of proteins than the Immobilon-PSQ, Immobilon-CD, Hyperbond, Fluorotrans, ProBlott, Transblot, and polypropylene membranes.
16. If longer time and higher temperature (60–80°C) were used, the deacetylation is more efficient, but high background level significantly decreases the sensitivity and make identification of amino acid impossible. The treatment with the *Pyrococcus* aminopeptidase under optimized conditions results in sufficient deblocking with initial yields up to 50%. However, the initial yields in the sequencer for some large proteins are below 5%.
17. When the sequence of the truncated polypeptide is analyzed, the *Pyrococcus* aminopeptidase itself is simultaneously sequenced. The N-terminal sequence of the aminopeptidase is MVDYELLKKVVEAPGV. The digest has the N-terminal sequence of Xaa-Pro-. If the sample on a glass fiber filter is treated with o-phthalaldehyde (OPA) after the first cycle of Edman degradation, the N-terminus of the *Pyrococcus* aminopeptidase is specifically blocked. However, the digest is not blocked, since the N-terminal residue is proline. Therefore, the sequence of only the digest can be determined from the second cycle ***(9)***.
18. Purified proteins can be digested directly with protease without purification by PAGE, and subjected to MS/MS analysis.
19. The deblocking techniques may be used in combination to make possible sequential deblocking and sequencing of unknown proteins immmobilized onto PVDF membranes (see **Fig. 2**).
    a. A protein sample is transferred from the PAGE gel onto a PVDF membrane by Western blotting.
    b. The membrane carrying the protein is directly subjected to gas-phase sequencing.
    c. If sequencing (2–3 cycles) fails at step a, the membrane is removed from the sequencer, and treated with TFA vapor at 60°C for 30 min to remove the acetyl groups of acetylserine and acetylthreonine. If the protein is N-terminally blocked by acetylserine or acetylthreonine, the acetyl group is removed by this procedure and sequencing from the N-terminus becomes possible.
    d. If sequencing fails at step b, the membrane is incubated in 0.6 M HCl at 25°C for 24 h to remove formyl groups, and then subjected to sequence analysis again.

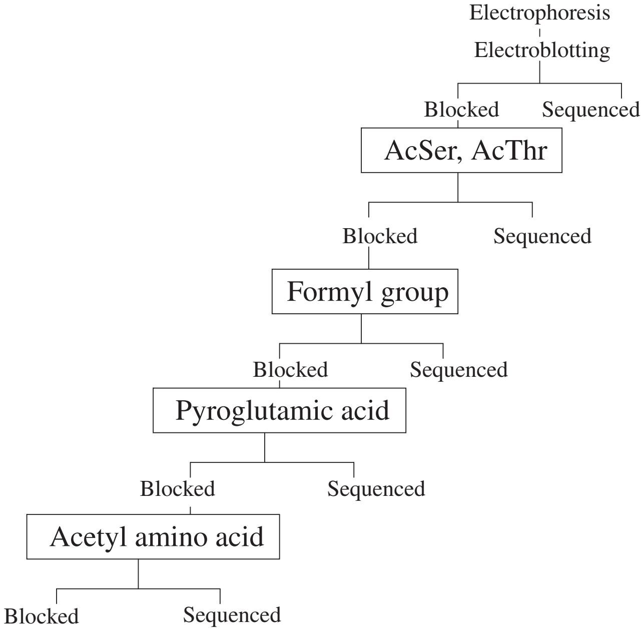

Electrophoresis
Electroblotting
Blocked
Sequenced
AcSer, AcThr
Blocked
Sequenced
Formyl group
Blocked
Sequenced
Pyroglutamic acid
Blocked
Sequenced
Acetyl amino acid
Blocked
Sequenced

Fig. 2. Strategy of sequential deblocking for N-terminally blocked proteins electroblotted on a PVDF membrane *(9)*.

e. If sequencing fails at step c, the sample is subjected to on-membrane pyroglutamyl peptidase digestion to remove N-terminal pyroglutamic acid and sequencing is again conducted.
f. Finally, if sequencing fails at step (d), deblocking with AARE is performed to remove acetylamino acids that are not removed in step (b) and sequencing is then attempted again. If sequencing still fails, different methods must be used.

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Deblocking of Proteins Containing N-Terminal Pyroglutamic Acid

Jacek Mozdzanowski

1. Introduction

Pyroglutamic acid (pGlu) is the product of cyclization of N-terminal glutamine and is one of the common modifications found at the N-termini of proteins. This modification is typical for immunoglobulins, especially IgGs, since they often contain glutamine at the amino terminus of the heavy chain. This chemical modification, in addition to other modifications of the N-terminus, results in a "blocked" protein, which cannot be sequenced using Edman degradation. Many deblocking procedures have been reported to remove common modifications at the N-terminal end of proteins for the sequence determination by Edman chemistry ***(1)***. Deblocking of N-terminal pGlu is usually achieved by the use of either chemical methods ***(2–5)*** or by the use of pyroglutamate aminopeptidase (PGAP) ***(1,6–8)***. Removal of pGlu from peptides by PGAP with high yield has been described ***(1,6–10)*** and recommended as a standard procedure ***(1).*** However, when this protocol is applied to large proteins such as IgGs, it results in relatively low yield (<40%) of the deblocked protein. The most commonly cited procedure of Podell and Abraham ***(11, 12)*** for removal of pGlu from proteins is also recommended in the handbooks ***(1,13,14)***. However, this procedure is not consistent with the results, which demonstrated that incubation at 4°C did not yield any deblocking ***(15)***. Other existing protocols use PGAP for digestion of proteins in solution or for proteins blotted onto PVDF membranes and result in variable yields ***(2,11,13,16,17,18,19)***. The use of thermostable PGAP from Pyrococcus furiosus allows to simplify the deblocking procedure and reduce the reaction time at the expense of incomplete deblocking, and possible partial degradation/fragmentation of the deblocked protein ***(19)***. The need to confirm cDNA coded protein sequence and to determine the purity of recombinant therapeutic

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proteins by reliable N-terminal sequencing requires highly efficient procedure that can remove N-terminal pGlu without protein fragmentation ***(15)***. This chapter describes such a procedure utilizing pyroglutamate aminopeptidase for deblocking of proteins in solution.

2. Materials

2.1. Reduction and Carboxymethylation of Proteins

1. Dithiothreitol (DTT)
2. 8 *M* guanidine buffer: 8 *M* guanidine, 0.35 *M* Tris-HCl, pH 8.5 (this buffer may be stored at room temperature for approx. 1 yr).
3. Iodoacetic acid, sodium salt

2.2. Desalting/Buffer Exchange

1. Sephadex G-25 column (NAP™-5, GE Healthcare, gel bed dimensions 0.9 × 2.8 cm).
2. Phosphate buffer: 0.1 *M* sodium phosphate, 2 m*M* EDTA, pH = 8.0. It is highly recommended that this buffer be prepared fresh for each experiment.

2.3. Digestion with Pyroglutamate Aminopeptidase

1. Glycerol
2. 1.0 M DTT solution in phosphate buffer (see **Subheading 2.2,** phosphate buffer). Prepare fresh for each experiment.
3. Pyroglutamate Aminopeptidase (Takara, Shuzo Co. Ltd., Code No. 7334).

3. Methods

3.1. Reduction and Carboxymethylation of Protein

1. Dissolve 0.5 mg of protein (e.g. IgG) in 1.0 mL of guanidine buffer (*see* **Note 1**).
2. Add 8.5 mg DTT and dissolve it in the reaction mixture.
3. Blanket the reaction mixture with argon.
4. Incubate at 60°C for 90 min.
5. Cool the reaction mixture down to room temperature.
6. Add 28 mg of solid sodium iodoacetate and dissolve in the reaction mixture (*see* **Note 2**).
7. Incubate at room temperature for 45 min in the dark (wrapped with aluminum foil) (*see* **Note 3**).
8. Add approx. 5 mg DTT and dissolve in the reaction mixture (*see* **Note 4**).

3.2. Desalting/Buffer Exchange

1. Equilibrate Sephadex G-25 (NAP-5) column with approx. 10 mL of 0.1 *M* phosphate buffer (0.1 *M* phosphate, 2 m*M* EDTA, pH 8.0) (*see* **Note 5**).
2. Load 0.3 mL of the reaction mixture containing reduced/carboxymethylated protein to the column (*see* **Note 6**).

3. Wait until there is no solution left above the top frit of the NAP-5 column. Discard the eluate (*see* **Note 7**).
4. Apply 0.55 mL of phosphate buffer (0.1 M phosphate, 2 mM EDTA, pH 8.0) to the column (*see* **Note 6**).
5. Wait until there is no solution left above the top frit of the NAP-5 column. Discard the eluate.
6. Place a clean vial under the column and apply 0.4 mL of phosphate buffer (0.1 *M* phosphate, 2 m*M* EDTA, pH 8.0) to the column.
7. Wait until there is no solution left above the top frit of the NAP-5 column. Collect the eluate (*see* **Note 8**).

3.3. Digestion with Pyroglutamate Aminopeptidase

1. Add 20 μL of glycerol to 0.4 mL of desalted reaction mixture (*see* desalting/buffer exchange, **Subheading 3.2**).
2. Add 5 μL of 1.0 *M* DTT solution in phosphate buffer (0.1 *M* phosphate, 2 m*M* EDTA, pH 8.0) to the reaction mixture (*see* **Note 9**).
3. Dissolve pyroglutamate aminopeptidase in 100 μL of phosphate buffer (0.1 *M* phosphate, 2 m*M* EDTA, pH 8.0) (*see* **Note 10**).
4. Add 10-20 μL of pyroglutamate aminopeptidase solution to the reaction mixture (*see* **Note 11**).
5. Blanket the reaction mixture with argon.
6. Incubate at 37°C for 24 h (*see* **Note 12**).
7. Sequence the sample (*see* **Note 13**).

4. Notes

1. Typically proteins are available as a solution. In most cases the protein solution may be mixed with the guanidine buffer to produce a final protein concentration of 0.5 mg/mL. Dilution of the guanidine buffer from 8 *M* to 4 *M* does not affect the reduction/carboxymethylation process. Proteins in dilute solutions may require concentration. The amount of protein needed for sequencing is much lower than 0.5 mg. This procedure may be modified for use with limited amounts of protein.
2. The amount of sodium iodoacetate has to be in stoichiometric excess of DTT in the reaction mixture. If the amount of DTT used in step 2 is greater than 8.5 mg, 28 mg of sodium iodoacetate may be insufficient for carboxymethylation. Sodium iodoacetate decomposes with time producing free iodine. Free iodine may result in iodination of tyrosines. Sodium iodoacetate which is pale yellow or yellow should not be used. Exposure to light increases the degradation of sodium iodoacetate.
3. Suggested incubation time for both the reduction and carboxymethylation steps is to assure a complete reaction. With many proteins a complete reaction may be achieved in a shorter time.

4. DTT is added to neutralize the unreacted iodocatetae. The exact amount of DTT added at this step is not critical.
5. Excess phosphate buffer (~10 mL) will provide very good equilibration of the NAP-5 column. The exact volume is not critical and larger volumes used for equilibration will not affect the procedure. If a desalting column of different volume is used, the volume used for equilibration should be equal to at least 3 column volumes.
6. All the volumes are given for Sephadex G-25 NAP-5 column from GE Healthcare. Other desalting columns may be used after the elution volume for a protein is established. The elution volumes may vary for different proteins. Low molecular weight proteins or peptides cannot be processed using the described desalting protocol. Certain proteins may bind to the resin and cannot be recovered from the desalting column (this is not common). The volumes given in this protocol are based on elution of IgGs.
7. The buffer flow through the column will stop as soon as there is no more liquid above the top frit. There is no danger of air getting inside the resin bed.
8. The method was not optimized for quantitative recovery of protein. This volume may require modifications for different proteins. If quantitative recovery is desired, the exact elution profile established by any suitable method (e.g., absorbance at 280 nm) will be very helpful.
9. Pyroglutamate aminopeptidase is a thiol protease and the presence of DTT is important for the activity of the enzyme.
10. Takara sells pyroglutamate aminopeptidase in lyophilized form, which is stable for several months under the proper storage conditions. After the enzyme is dissolved, it should be stored frozen (-70°C) and used within 1 month.
11. The amount of PGAP given here was optimized for IgGs. Larger amount of PGAP will improve deblocking yield. Commercially available enzyme is highly purified and the use of larger amount will have minimal effect on sequencing background.
12. Pyroglutamate aminopeptidase from *Pyrococcus furiosus* (Takara) can be used at higher temperatures. Higher incubation temperature reduces the time necessary for digestion but often increases protein degradation and results in higher background in sequencing. In some cases incubation at 37°C for 2 or 3 d may be beneficial for the deblocking yield.
13. This procedure was used for the deblocking of several IgGs. There is no reason to anticipate problems when deblocking other proteins. In general the expected deblocking yield is between 80-100%.

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Detection and Characterization of Protein Mutations by Mass Spectrometry

Yoshinao Wada

1. Introduction

A significant proportion of genetic disorders are caused by point mutations in proteins. Although most structural studies of mutated proteins are carried out by genetic methods, mass spectrometry (MS) is still a more rapid way of the analysis if sufficient amounts of proteins are obtained. The process for characterization, *i.e*. finding aberrant signals in the mass spectrum and determining the site and nature of mutations, described here is applicable to recombinant proteins and to the structural studies of post-translational modifications.

The principal part of analysis includes a method called "peptide mass mapping" which aims at both detection and characterization of mutations, and employs electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) for ionization of peptides. In addition, these soft ionization methods have allowed MS to deal with whole protein molecules for detection of mutations, and this approach is effective for the proteins of molecular mass <20,000-50,000, depending on the resolving power of mass analyzers ***(1-4)***.

For the characterization of mutations in the peptides of interest, collision-induced dissociation (CID), or tandem MS (MS/MS), is successful in most cases, because the product ion mass spectra can be read in comparison with the normal reference ones.

Summary of procedures

1. (prior to MS) Purification. alkylation, and desalting.
2. Detection of variants through mass measurement of the intact protein molecules.
3. Chemical or enzymatic cleavage.
4. Peptide mass mapping to identify mutated peptides.
5. Data interpretation.

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2. Materials

2.1. Apparatus for Sample Preparation

1. Desalting tools using reversed phase media such as ZipTip (Millipore): ZipTipC4 for proteins and ZipTipC18 for peptides.
2. NAP-5 (GE Healthcare)

2.2. Chemicals

1. Dithiothreitol (Sigma).
2. Iodoacetic acid (Sigma) or iodoacetamide (Sigma).
3. Cyanogen bromide (CNBr) (Sigma) (toxic, handle with gloves in hood).
4. Acetonitrile (HPLC gradoemt grade; Merck).
5. Formic acid (reagent grade; Merck).
6. Trypsin (TPCK-treated) (Sigma type XIII).
7. Lysylendopeptidase (Wako, Osaka, Japan).
8. Endoproteinase Glu-C (Roche).
9. Endoproteinase Asp-N (Roche).
10. Solutions for reduction and alkylation: Stock solution A: 1 *M* Tris-HCI containing 4 m*M* EDTA, adjusted to pH 8.5 with HCl. Stock solution B: 8 *M* guanidium chloride. Mix stock solutions A and B at a 1:3 ratio to make a solution of 6 *M* guanidium chloride, 0.25 *M* Tris-HCl, and 1 m*M* EDTA prior to use.
11. 0.05 *N* HCl.
12. 1.5 *M* Tris.
13. 50 m*M* Ammonium hydrogen-carbonate, pH 7.8 (pH adjustment is not required).
14. 50 m*M* Ammonium carbonate, pH 8.3 (pH adjustment is not required).
15. 50 m*M* Tris-HCl, pH 7.4.
16. 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic aicd, 10 mg/mL) in acetonitrile: water:TFA (30:69.9:0.1 [v/v/v]) for MALDI matrix for proteins; 2,5-dihydroxy-benzoic acid (10mg/mL) or α–cyano-4-hydroxycinnamic acid (saturated solution) in acetonitrile:water:TFA (50:49.9:0.1 [v/v/v]) for MALDI matrices for peptides.
17. Acetonitrile:water:acetic acid (49:49:2 [v/v/v]) or methanol:water:formic acid (70:29.9:0.1 [v/v/v]) for ESI solvent.
18. Glycerol:trichloroacetic acid (95:5 [w/w]) for FAB matrix.

3. Methods

3.1. Purification and Desalting for MS

Removing salts (desalting) is preferred because the formation of adduct ions with alkali metals such as sodium and potassium reduces the abundance of the protonated molecular ion species, which are the informative ions for the mass measurement of proteins and peptides, and unnecessarily complicates mass spectra. In order to prevent this, RP-LC is recommended as the final step of purification procedures. Alternatively, a more convenient method of desalting is to use a pipette tip with a bed of chromatography media such as ZipTip according to the manufacturer's protocol shortly as follows.

1. For pre-wetting ZipTip, aspirate 50% methanol (wetting solution) into tip. Dispense to waste and repeat.
2. Equilibrate the tip for binding by washing with the equilibration solution (0.1% TFA) 3 times.
3. Bind peptides or proteins, which are dissolved in acidic solution such as 0.1%–1.0% TFA without organic solvents, to ZipTip by fully depressing the pipettor plunger to a dead stop. Aspirate and dispense sample 3 to 7 cycles for simple mixtures and up to 10 cycles for maximum binding of complex mixtures.
4. Wash tip and dispense to waste using at least five cycles of wash solution. A 5% methanol in 0.1% TFA/water wash can improve desalting efficiency.
5. Dispense 5 to 10 µL of elution solution (75% methanol / 0.1% TFA or 0.1% formic acid) into a clean vial using a standard pipette tip. Carefully, aspirate and dispense eluate through ZipTip at least three times without introducing air.
6. For ESI MS, the sample can be eluted into clean vial or into a nanospray needle for direct infusion MS, for which the elution solution in step 5 should not contain TFA. For MALDI MS, an aliquot of eluate is mixed with the same volume of sinapinic acid solution in a clean vial, and then placed on a sample target and dried.

3.2. Detection of Variants Through Mass Measurement of the Intact Protein Molecule with MS

Among the naturally occurring amino acids, Leu and lle have the same elemental composition. Lys and Gln are different in composition, but have the same nominal mass. All other amino acids differ from each other in their molecular mass by ≤129 Da. Variants having substitutions of these amino acids are different in molecular mass from the corresponding normal proteins and are thus theoretically detectable by measuring the molecular mass of the intact protein by MS.

For ESI-MS, the sample at a concentration of 1–10 µ*M* in the ESI solvent is introduced into the ion source. Any sample remaining in the delivery tube after analysis is recovered and lyophilized for further analysis, if necessary.

Fig. 1 shows the MALDI-TOF mass spectrum of the globins from a patient with a mutant hemoglobin. Two different β-globin species are clearly differentiated in the mass spectrum taken at a resolution of 3,000, and the mass separation was determined from this analysis to be 14 u (*see* **Note 1**).

3.3. Chemical or Enzymatic Cleavage

3.3.1. Reduction and Alkylation

Cysteine residues are often subject to auto-oxidation during preparation procedures, leading to a variety of products and random formation of disulfide bridges during preparation procedures. To avoid these unexpected modifications, conversion to stable derivatives by alkylation should be performed after reduction. Such a modification destroys the higher order structure of the protein by cleaving intra- or inter-molecular bridges, thus facilitating the action of proteolytic enzymes by allowing them to access cleavage sites.

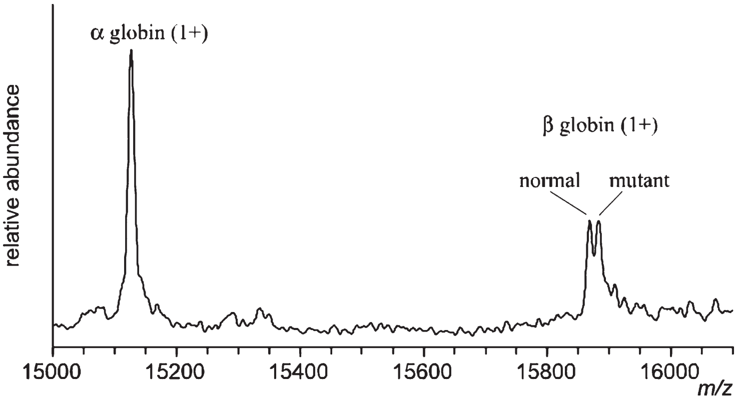

Fig. 1. Detection of a variant protein by MALDI-TOF-MS. The mass separation is determined to be 14 u.

1. Dissolve the protein at 1–20 µg/mL in a 400 µL sample buffer containing 6 *M* guanidium chloride, 0.25 *M* Tris-HCl and 1 m*M* EDTA, pH 8.5.
2. Add 5 µL of 1 *M* dithiothreitol (freshly prepared before use) dissolved in sample buffer directly and incubate under nitrogen gas at 50 °C for 30 min.
3. Chill down the tubes to room temperature.
4. Add 50 µL of 1 *M* iodoacetamide in sample buffer or iodoacetic acid in 0.1 *N* NaOH, and incubate for 30 min at room temperature in the dark.
5. Make up the volume to 500 µL with water.
6. Transfer onto a NAP-5 column equilibrated with 0.05 *N* HCl, and recover the proteins in a subsequent 1 mL solution of 0.05 *N* HCl.
7. Proteins are lyophilized or are subjected to enzymatic digestion after addition of 1/10 volume of 1.5 *M* Tris to raise the pH above 8.0.

3.3.2. Cleavage with CNBr (Optional)

CNBr cleaves only at methionine residues in proteins, generating large peptide fragments compared with enzymatic cleavage. The peptides will be <20,000 Da, which is well within the range that can be measured precisely by MS. Most of the resulting C-terminal homoserine residues of the peptides from this type of cleavage are in lactone form. Cleavage by CNBr helps to confirm a suspected site of mutation to a small part of the protein molecule and for determining the size of the molecular shift precisely.

1. Dissolve the protein at 1–20 µg/mL in 70% (v/v) TFA (*see* **Note 2**).
2. Add a >100-fold molar excess, over methionine residues, of CNBr dissolved in 70% TFA solution, and incubate in the dark under nitrogen at room temperature for 2 h.
3. Add 10 vol of water, and remove salts as described in **Subheading 3.1.**
4. ESI- or MALDI-MS, as described in **Subheading 3.1.**

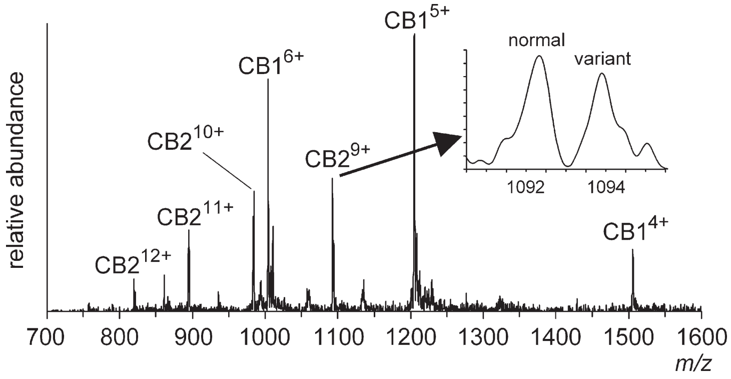

Fig. 2. ESI mass spectrum of CNBr peptides of the β-globin isolated from the same patient shown in **Fig. 1.** The region of the nine-charged ion for peptide CB2 is depicted in an inset. A Cys-containing peptide CB2 is detected at the expected mass without alkylation. The resolution is 2,000.

In the example shown in **Fig. 2**, a mutation causing a difference of 14u is identified in peptide CB2 (M_r 9821.4 for the normal species) of β-globin. The separation of the normal and the variant species is obviously better than that of the intact proteins shown in **Fig. 1**.

3.3.3. Enzymatic Digestion and Peptide Mass Mapping

The following enzymes are amino acid-specific in their cleavage, and are employed for peptide mass mapping: trypsin, lysylendopeptidase (endoproteinase Lys-C), endoproteinase Glu-C, endoproteinase Asp-N. Using volatile buffers allows to omit the desalting process prior to peptide mass mapping. Alternatively, desalting can be performed as described in **Subheading 3.1.**

1. Dissolve proteins in the digestion buffer at about 1–20 µg/mL.
2. Optimized buffers: for trypsin and endoproteinase Glu-C, 50 m*M* ammonium hydrogen-carbonate (pH 7.8); for endoproteinase Lys-C, 50 m*M* ammonium carbonate (pH 8.3); for endoproteinase Asp-N, 50 m*M* Tris-HCl (pH 7.4) (*see* **Note 3**).
3. Add protease at >1% (w/w) ratio and digest at 37°C for 6 h.
4. Remove salts according to the procedure in **Subheading 3.1**, or lyophilize directly if volatile buffer is used.
5. ESI or MALDI MS, as described in **Subheading 3.1.**

3.4. Peptide Mass Mapping

The mass spectra of a tryptic digest of β-globin by ESI, MALDI and fast atom bombardment (FAB) are shown in **Fig. 3**. The coverage is the same between ionization methods. ESI MS of peptides can be directly forwarded to the succeeding

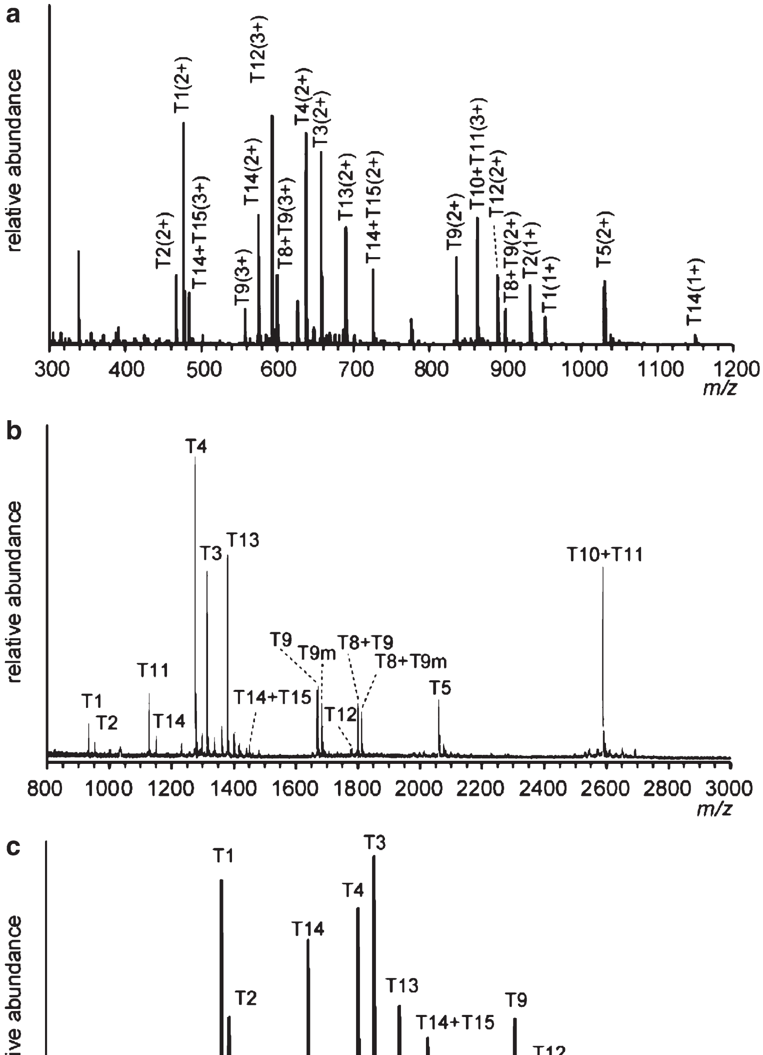

Fig. 3. Mass spectra of the tryptic digest of carboxymethylated β-globin acquired by different ionizations, **(a)** ESI, **(b)** MALDI and **(c)** FAB. The number above each peak is that of the predicted tryptic peptide starting from the N-terminus. In the MALDI-TOF mass spectrum, mutated peptides, T9m and T8+T9m, are detected beside the corresponding normal peptides **(see Fig. 8a).**

CID for sequence analysis, though the mass spectrum is complicated due to a number of multiply charged ions from the component peptides. LC/MS can be operated with ESI, facilitating detection of component peptides with good signal-to-noise ratio. MALDI-TOF produces a lucid mass spectrum comprised of singly charged ions, but the relative intensity of ions is not correlative with the abundance of the corresponding peptides **(Fig. 3b)**. FAB ionization presents a simple mass spectrum as MALDI but is less sensitive than other ionizations **(Fig. 3c)**.

3.5. Characterization of the Mutation

Categorized strategies are described here using examples of variant hemoglobins (*see* **Note 4**).

3.5.1. Simple Cases

1. New cleavage site: The example shown in **Fig. 4** is a hemoglobin variant with a mutation in α-globin. Tryptic peptide 6 (T6), which should be detected at *m/z* 1833.9. is missing, and a new peptide appears at *m/z* 1634.8 in the mass spectrum. The substitution of Arg for Gln at position 54 is the only possible substitution that fits the molecular mass change. The substitution can be verified by a digestion with lysylendopeptidase, which does not cleave arginyl peptide bonds; the peptide ion is shifted from the normal *m/z* 1833.9 to *m/z* 1862.0, with the increase of 28 u confirming the Gln→Arg mutation.

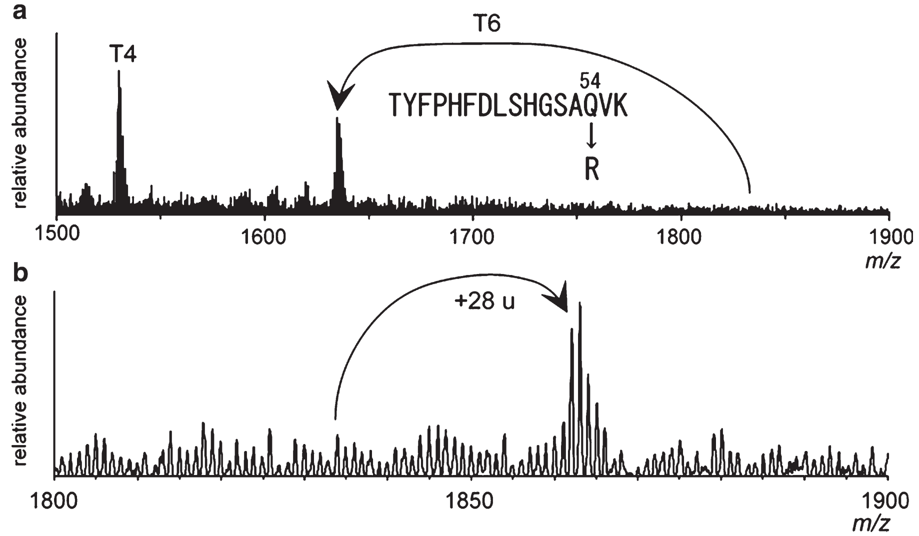

Fig. 4. FAB mass spectra of (**a**) tryptic and (**b**) lysylendopeptidase digests of an α-globin variant.

2. Missing cleavage site: In **Fig. 5**, a β-globin variant, tryptic peptides 13 (T13) and 14 (Tl4) are missing in the digest, indicating a mutation at the Lys residue at position 132. The new peptide is observed at *m/z* 2495.3, indicating the replacement of Lys by Asn.
3. Unique replacement: In **Fig. 6,** tryptic peptide 11 (T11) at *m/z* 1126.6 is missing (a trace due to insufficient separation from normal protein) and a new peptide appears at *m/z* 1152.6 in the digest of purified β-globin of the variant hemoglobin. This increase of 26 u suggests five possible mutations; His→Tyr, Ala→Pro, cmCys (carboxamidomethylcysteine)→Trp, Ser→Leu, and Ser→Ile. Since the normal peptide has His, but not Ala, Cys, or Ser residues, and has a single His residue at position 97 in the sequence, it follows that the latter has been replaced by Tyr.

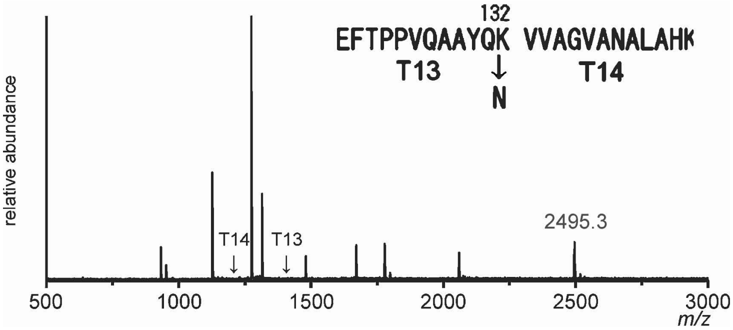

Fig. 5. MALDI mass spectrum of the tryptic digest of a β-globin variant.

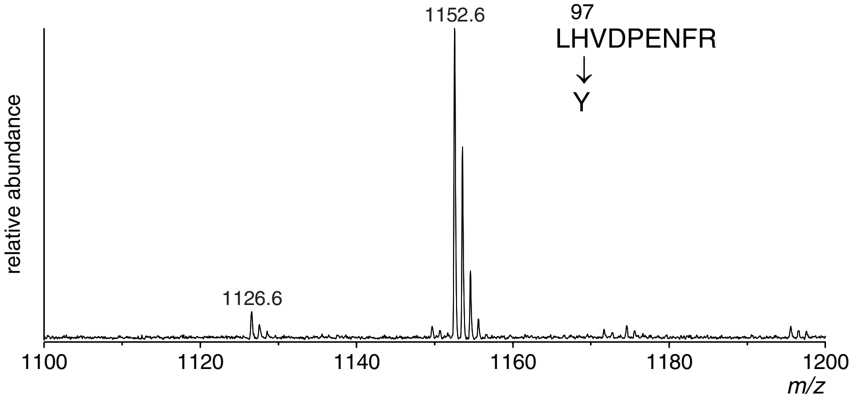

Fig. 6. MALDI mass spectrum of the tryptic digest of a β-globin variant. A trace peak at *m/z* 1126.6 is derived from contamination of normal protein.

3.5.2. Precise Measurements with High-Resolution MS

High-resolution MS that can determine the mass of peptides at least to the third decimal place is available with a Fourier transform ion cyclotron resonance (FTICR) or an orbitrap mass analyzer. The technology enhances the capacity of the peptide mass mapping strategy for structural characterization *(5)*. For the same mutation shown in **Fig. 6**, five types of substitutions are suggested, and the corresponding mass shifts in exact mass are as follows: His→Tyr (26.0044), Ala→Pro (26.0157), cmCys→Trp (26.0487), Ser→Leu (26.0520), and Ser→Ile (26.0520). FTICR mass spectrum shown in **Fig. 7** is capable of discriminating a subtle difference in these mass shifts ocurring in a 1 kDa peptide, and the statistics from 20 repeated measurements uniquely determines the type of substitution His→Tyr for the measured mass shift of 26.00439 ± 0.00046.

3.5.3. Complex Cases

In cases where the molecular weight of the mutated peptide does not permit the assignment of a unique type and/or position of substitution, chemical data, such as the charge carried on the variant, may provide an answer. Alternatively, combination of different enzymes may give conclusion. For an example shown in **Fig. 8**, the mass spectrum of a tryptic digest of β-globin from the patient discloses new peaks near the normal peptide 9 (T9), suggesting that the mutation must be

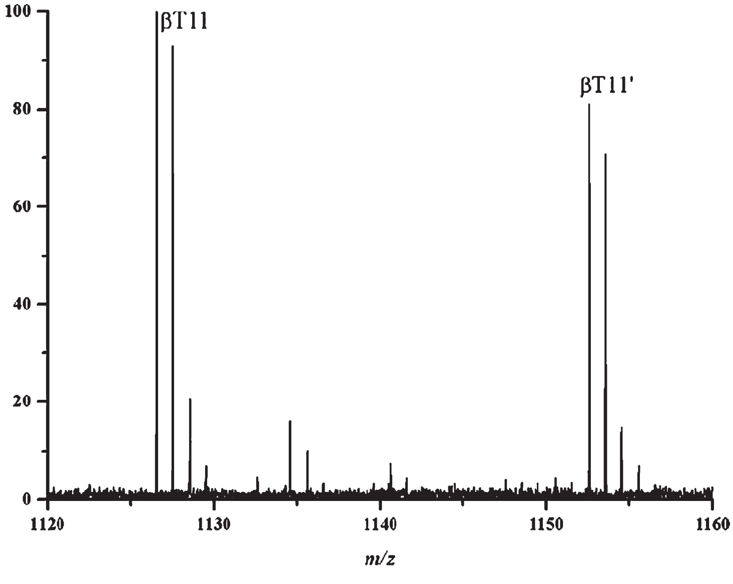

Fig. 7. FTICR mass spectrum of the tryptic digest of a β-globin variant (the same sample for Fig. 6. Reproduced from ***ref. 5*** under permission.

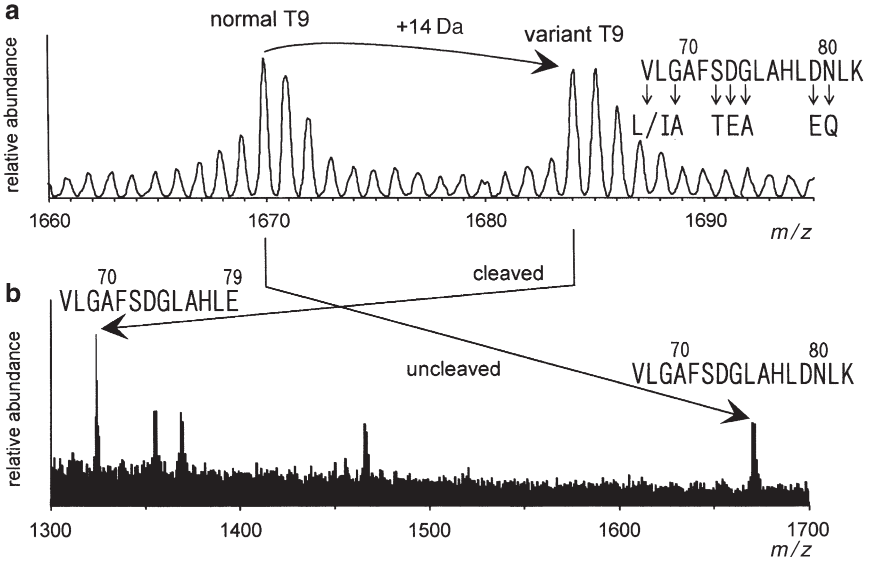

Fig. 8. FAB mass spectra of (**a**) tryptic and (**b**) endoproteinase Glu-C digests of a β-globin variant. The protonated molecular ions at *m/z* 1669.9 and at *m/z* 1683.9 are derived from the normal and variant proteins, respectively. The increase of 14 u for this peptide gives several possible types of mutations even with supporting chemical data. The substitution Asp79→Glu is determined by endoproteinase Glu-C digestion. See also **Fig. 10,** for CID analysis for verifying the structure.

in T9. The difference of 14 u suggests seven possible substitutions: Gly→Ala, Ser→Thr, Val→Leu, Val→Ile, Asn→Gln, Asp→Glu, Asn→Lys, and Thr→Asp. However, even on the basis of both the normal sequence and the electrophoretic data, six different substitutions at seven possible positions are left as candidates. In this instance, the substitution of Glu for Asp at position 79 is determined, when the tryptic digest is then hydrolyzed with endoproteinase Glu-C.

3.6. CID or Tandem Mass Spectrometry

In all the cases described above, it is necessary to determine the suggested mutation by CID or tandem MS. It is usually easy to read the CID mass spectrum, because the product ions from the mutated peptide can be compared with those from the normal one. Examples of the CID mass spectrum for mutation analysis based on ESI and MALDI and low-energy collision are presented in **Figs. 9** and **10**, respectively. High-energy collision may produce key fragments, especially when discrimination of Ile and Leu is required *(6)*. The CID analysis of intact proteins without digestion is an issue of challenge, while the information is still confined to a part of the molecule *(7)*.

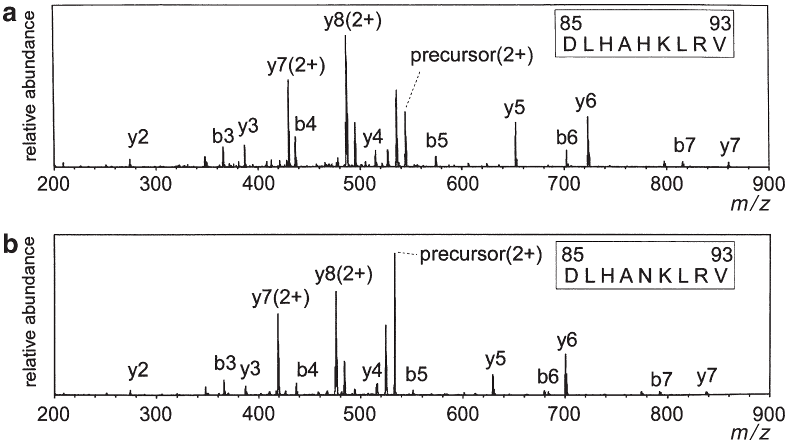

Fig. 9. CID mass spectra taken for the structural characterization of a mutated peptide by ESI ion trap MS. (**a**) As a reference, the $[M+2H]^{2+}$ ion for the normal peptide at *m/z* 544.8 was selected as the precursor. (**b**) The $[M+2H]^{2+}$ ion for the mutated peptide at *m/z* 533.3 was selected as the precursor. Note the difference in the *m/z* values for the b5 or the y5 product ion between the two spectra, indicating the mutation at position 89.

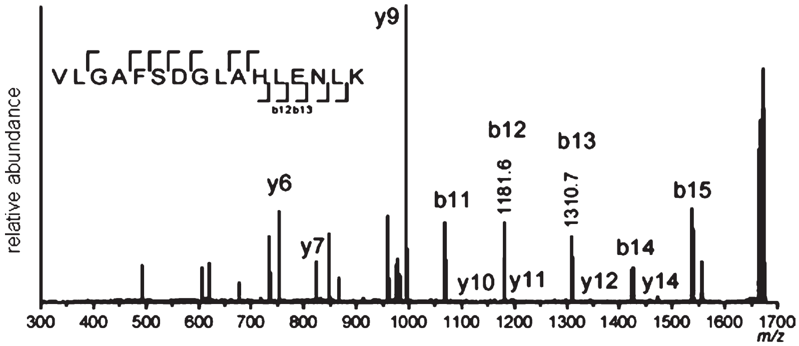

Fig. 10. CID mass spectrum of the ions for T9m (*m/z*1683.9) in **Fig. 3b.** The product ions at *m/z*1181.6 (b12) and *m/z*1310.7 (b13) indicate Glu for the 13th residue (position 79 in **Fig. 8a**) from the N-terminus of this peptide.

When mutations are suggested by MS of intact proteins or, preferably, the target sites are localized within specific peptides by peptide mass mapping, DNA sequence analysis can be efficiently applied to the corresponding gene for characterization of the responsible mutation *(8)*.

4. Notes

1. The theoretical isotopic distributions of human β-globin (146 residues, M_r 15,867) at resolutions of 15,000 (A) and 2,000 (B) are shown in **Fig. 11**. In actual measurements of proteins of molecular mass over several kilodaltons, it is usually impossible to obtain the high ion current necessary for unit resolution as (A), nor is it possible to identify the ^{12}C species corresponding to the exact mass in the molecular ion cluster. Consequently, the average mass of the unresolved isotopic cluster or "chemical mass" is usually used. The smallest difference in molecular masses detectable by MS taken at a modest resolving power of a few thousand increases with the increasing size of the proteins. A theoretical analysis can be considered using two model proteins: β-globin (M_r 15,867) and transferrin (M_r 79,556) **(Fig. 12)**. A separation of 14 u in transferrin is not clear (C in **Fig. 12b**), whereas the shapes of the clusters for 20 and 31 u differences, corresponding to D and E, respectively, in **Fig. 12b**, are similar to those for 9 and 14 u differences, corresponding to C and D, respectively, of the β-globin in **Fig. 12a**. The minimum difference in molecular mass detectable as pattern D in **Fig. 12b** for an equimolar mixture with normal proteins of M_r over 100,000 is about 20 u. This means that about half of the possible amino acid substitutions in a protein of this size will escape detection. In those cases, a strategy to detect these substitutions preferably starts with the analysis of CNBr-cleaved peptides **(Subheading 3.3.2)**.

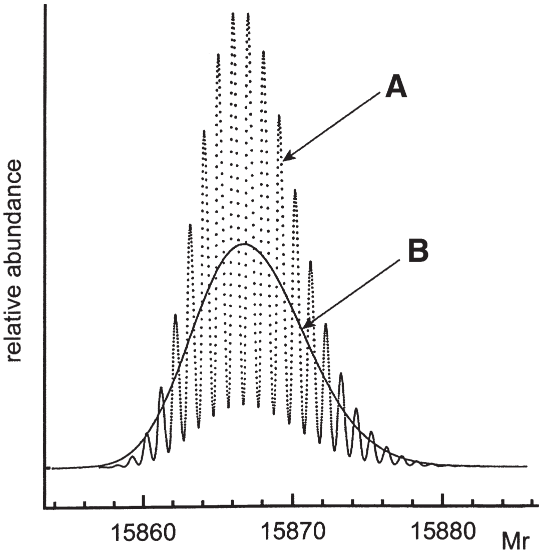

Fig. 11. Theoretical isotopic distributions of β-globin at resolutions of 15,000 **(A)** and 2,000 **(B)** (10% valley definition).

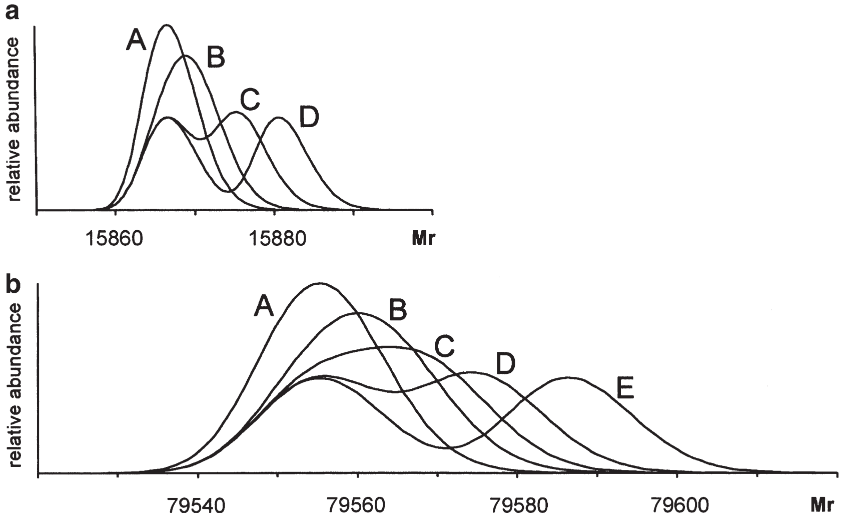

Fig. 12. Theoretical distributions of isotopic clusters corresponding to an equimolar mixture of **(a)** normal and variant β-globins. (A) Pure normal protein; (B) normal and +4 mass unit mutant; (C) normal and +9 mutant; (D) normal and +14 mutant. **(b)** Normal and variant transferrin. (A) Pure normal protein; (B) normal and +9 mass unit mutant; (C) normal and +14 mutant; (D) normal and +20 mutant; (E) normal and +31 mutant. The curves have been drawn by connecting the peak top of each isotopic ion from a display at sufficient resolution to achieve complete separation of ^{13}C isotopic peaks.

2. Cleavage in formic acid is not recommended, because it has a tendency to formylate amino and hydroxyl groups, which creates a mixture consisting of products differing in molecular mass by multiples of 28 Da.
3. Lysylendopeptidase is active in urea solution at a concentration up to 4 *M*. The digestion is often carried out in a solution of 4 *M* urea, 0.1 *M* Tris-HCl, pH 9.0, in which substrates are denatured and become susceptible to the cleavage.
4. Protein variants are heterozygous in most cases, namely both normal and mutated proteins are present in the patients as shown in **Fig. 1**. In this chapter, variant proteins, isolated from their normal counterpart, are presented, except for the cases of **Figs. 7** and **8.** *See* **refs. 7** and **8**.

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Peptide Sequencing by Nanoelectrospray Tandem Mass Spectrometry

Ole Nørregaard Jensen and Matthias Wilm

1. Introduction

Electrospray ionization mass spectrometry (ESI-MS) *(1)* has had a profound influence on biological research over the last decade. With this technique, it is possible to generate and characterize gas-phase analyte ions from aqueous solutions of proteins, peptides, and other classes of biomolecules. ESI is performed at atmospheric pressure, which simplifies sample preparation and handling and allows on-line coupling of chromatography, such as capillary high-performance liquid chromatography (HPLC), to mass spectrometers (LC-MS). The use of ESI in combination with tandem mass spectrometry (MS/MS) provides the capability for amino acid sequencing of peptides. The optimization and miniaturization of peptide sample preparation methods for ESI as well as the development of highly sensitive tandem mass spectrometers, including ion traps and quadrupole-time-of-flight (Q-TOF) and linear ion trap-fourier transform MS hybrid instruments, makes it possible and almost routine to obtain amino acid sequences from subpicomole levels of protein in many laboratories. Peptide sequencing is typically performed by nanoelectrospray MS/MS analysis of crude, concentrated peptide mixtures or by hyphenated techniques, such as capillary HPLC coupled to micro/ nanoelectrospray-MS/MS. The sets of peptide tandem mass spectra generated in such experiments are used to query biological sequence databases with the aim to identify all protein components present in the sample. In this chapter, we describe practical aspects of nanoelectrospray mass spectrometry aimed at amino acid sequencing of peptides at subpicomole levels. We do not consider LC-MS/MS, although many of the features of the two analytical approaches are very similar.

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1.1. The Nanoelectrospray Ion Source

The main characteristics of the nanoelectrospray ion source are low flow rate, high ionization efficiency, and extended measurement time with concomitant improvements in absolute sensitivity ***(2,3)*****.** These features are crucial for peptide sequencing by tandem mass spectrometry. Nanoelectrospray tandem mass spectrometry is a reliable and robust technique for identification or sequencing of gel-isolated proteins available in sub-picomole amounts ***(4,5)*****.** The very low flow rate of 10–25 nL/min of the nanoelectrospray source has required a change of sample preparation method from online HPLC separation to sequencing directly from desalted/concentrated peptide mixtures as outlined.

For nanoelectrospray tandem mass spectrometry and other mass spectrometric methods it is important to choose the most suitable protease for generation of peptides from a protein. Trypsin has several advantages for the generation of peptides for tandem mass spectrometric sequencing. It is an aggressive and extremely specific protease that cleaves the carboxy-(C)-terminal amide bond of lysine and arginine residues. Tryptic peptides typically have a mass between 800 Da and 2500 Da and both the amino-(N)-terminal amino group and the C-terminal residue (Lys or Arg) are basic so they generate mainly doubly charged peptide ions in electrospray. Such ions predominantly fragment at the peptide amide bond to generate singly charged N-terminal (b-type) or C-terminal (y-type) ions ***(6,7)*****.** Tryptic peptides rarely contain internal arginines that generate complicated fragmentation patterns. The in-gel digestion procedure ***(5,8)*** is compatible with matrix-assisted laser desorption/ionization mass spectrometry (MALDI) MS as well as nanoelectrospray mass spectrometry. In the latter case a sample desalting/concentration step is necessary (Protocol 2 or 3)

1.2. Analytical Characteristics of the Nanoelectrospray Ion Source

The flow rate of a nanoelectrospray source is about 25 nL/min. At this flow rate an analyte concentration of 1 pmol/µL results in one analyte molecule per droplet on the average ***(2)*****.** Furthermore, 1 µL of sample is consumed in 40 min extending the time available for optimization of experiments. The overall sensitivity is limited by the signal-to-noise level and it is therefore a function of the ionization efficiency, desolvation efficiency, ion transmission, the level of chemical background ions, and detector characteristics.

The electrospray generated with the nanoelectrospray ion source is very stable. This allows purely aqueous solutions to be sprayed even in negative ion mode without nebulizer assistance. The source can be operated with solutions containing up to 1 *M* NaCl ***(3)*****,** although this is not recommended. The high stability allows optimization of experimental conditions based on analyte characteristics rather than electrospray requirements, that is when choosing buffer composition.

For example, preservation of noncovalent complexes often does not allow addition of organic solvent to the sample to facilitate spraying. Because the ion source exclusively produces very small droplets, relatively soft desolvation conditions in the interface region of the mass spectrometer can be chosen.

The nanoelectrospray source consists of a metal-coated glass capillary whose tip is pulled into a needle shape with an outer diameter of about 2 µm and an orifice diameter of 1 µm **(Fig. 1)**. The glass capillary is mounted in a gas-tight holder that can be pressurized by air up to about 1 bar **(Fig. 2)**. The needle assembly is connected to the ion source power supply. Holder and needle are electrically connected, for example, by a small droplet of conductive carbon cement (Neubauer Chemikalien, Münster, Germany). The metal coating of the glass capillary needle ensures that the electrical potential is transferred to the liquid sample at the needle tip.

The nanoelectrospray needles are made from borosilicate glass capillaries (GC 120 F–10, Clark Electromedical Instruments, Pangbourne, UK). The desired needle shape is obtained by using a two-stage pulling cycle on a microcapillary puller (Model P-97, Sutter Instruments Co., USA; parameter set [heat, pull, velocity, time]: 1—520, 100, 10, 200; 2–490, 160, 12, 165. The first heating/pulling stage reduces the diameter of the capillary to about 0.5 mm, while the second stage pulls the glass capillary apart, producing two nanoelectrospray needles. These needles should have an opening of 1–2 µm. However, after pulling the opening diameter can be less than 100 nm and has to be widened (*see* Methods). Nanolectrospray needles and ion sources are commercially available from New Objective (Boston, MA) and Proxeon Biosystems (Odense, Denmark) as well as from several MS instrument manufacturers.

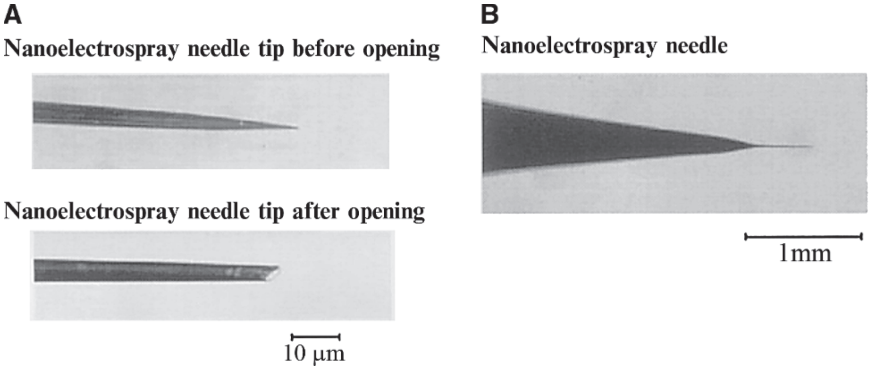

A
Nanoelectrospray needle tip before opening
Nanoelectrospray needle tip after opening
10 µm
B
Nanoelectrospray needle
1mm

Fig. 1. Nanoelectrospray needles. Pulled glass capillary needle tip before (**A**) and after (**B**) opening. The needle tip should be 200–500 µm long with an opening of only 1–2 µm. (**C**) A two-stage heat/pull cycle on a capillary puller produces two identical glass capillary needles with a thin, tapered needle tip.

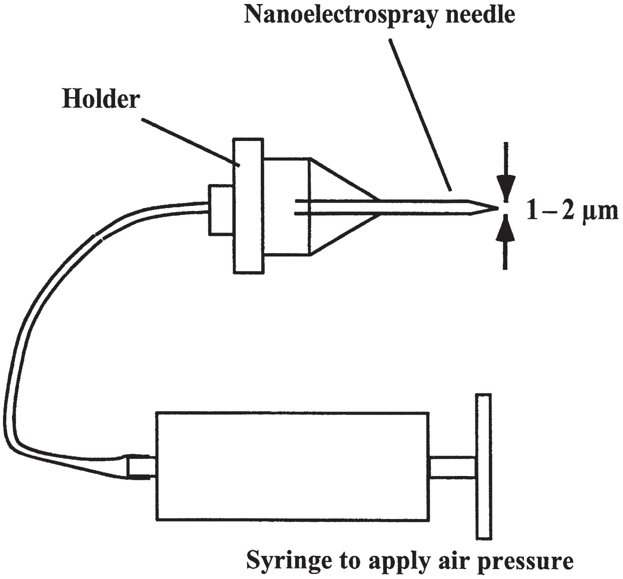


Fig. 2. Nanoelectrospray ion source. The nanoelectrospray needle is mounted in a metal holder that is connected to the ion source power supply and to a syringe that provides the air back pressure. The needle assembly is mounted onto a *x-y-z* manipulator and positioned on axis and 1.5–2 mm from the orifice of the mass spectrometer.

Liquid injected into the needle is drawn to the tip by capillary force. To reduce the flow resistance for a stable flow rate in the 10–25 nL/min range the narrow part of the tip should not be longer than 500 µm (**Fig. 1**). Needles with very short constrictions (50–100 µm) can be operated easily but with a higher risk of losing sample due to a higher flow rate. Short needles are preferred for rapid mass measurements when abundant sample is available, for example, recombinant proteins, synthetic peptides, or oligonucleotides. Longer tips (200–500 µm) are used for tandem mass spectrometry experiments when the longest possible operation time is desirable and when the sample load volume will not exceed 1 µL. A major advantage of these types of nanelectrospray needles is that they do not easily block due to the relatively short length of the needle tip.

Metal coating of the glass capillaries is achieved by metal (gold) vapor deposition in a sputter chamber (Polaron SC 7610 sputter coater, Fisons Instruments, East Sussex, UK). The needles are used only once so it is not a problem that the coating is not tightly fixed to the glass and can be rubbed off. Methods to produce a more stable metal coating include pretreatment with (3-mercaptopropyl) trimethoxysilane ***(9)*** or protecting the metal layer by a second layer of SiO_x ***(10)***. A stable gold coating is necessary when a glass needle is used for several samples over a prolonged time. As the needle tip is fragile it should be handled carefully when loading the sample and when mounting it in the holder.

2. Materials

2.1. Sample Desalting and Concentration Employing Glass Capillary Microcolumns

1. Chromatographic resin (e.g., Poros R1, R2, or Oligo R3).
2. Pulled glass capillaries for microcolumns and nanoelectrospray needle.
3. Custom-made holder for centrifugal loading and transfer of sample from capillary column to nanoelectrospray needle (available from Proxeon Biosystems, Odense, Denmark).

2.2. Sample Desalting and Concentration Employing Microcolumns Packed in GELoader Tips

1-mL plastic syringe; GELoader tips (Eppendorf brand). The syringe is adapted to the GELoader tips by using the top part of a "yellow" plastic tip.

3. Methods

3.1. Operation of the Nanoelectrospray Ion Source

Nanoelectrospray ion sources are now optional on a number of electrospray mass spectrometers or they can be custom made for individual instruments. Note that electrospray ion sources are operated at high voltages and care should be taken to avoid electrical shock.

1. *The geometrical location of the ion source*. The nanoelectrospray source is mounted directly in front of the orifice of the mass spectrometer, usually at a distance of 1.5–2 mm from the orifice. Electrospray at a low flow rate generates very small droplets with a diameter of 200 nm or less. Desolvation is therefore achieved in a very short time and distance.
2. *The voltage applied to the source*. To initiate the electrospray a minimal electrical field strength at the surface of the liquid has to be reached ***(11)***. Conventional electrospray ion sources are operated with a 3–5 kV potential difference between the needle and counterelectrode (i.e., the orifice plate of the mass spectrometer). The very small tip diameter of the nanoelectrospray needle allows a spray cone to be established at a much lower electrical potential, typically 500–900 V.
3. *The desolvation conditions in the interface region*. Because the charged droplets generated by the nanoelectrospray ion source are very small, softer desolvation conditions in the interface (skimmer) region of the mass spectrometer are used. The electrical gradient and the countercurrent gas flow can be reduced. This appears to lead to the generation of colder molecular ions as compared to conventional electrospray sources, facilitating, for example, studies of noncovalent molecular interactions. The main objective when operating the nanoelectrospray source is

to achieve a low and stable flow rate despite sample-to-sample variations. This is achieved by applying air pressure to the needle which helps to adjust the flow rate and thereby compensates for differences in needle orifice size and sample viscosity.

A step-by-step guide for installing the nanoelectrospray source is given in the following list. The order of events may not be compatible with all nanoelectrospray ion sources:

1. Mount the pulled and metal coated nanoelectrospray needle in the holder. Connect the holder to the ion source power supply.
2. Make electrical contact between the needle and the holder, for example, by applying a droplet of graphite paste. (Alternatively, the power supply can be connected directly to the needle with a clamp.)
3. Mount the ion source on an x-y-z manipulator in front of the mass spectrometer.
4. Inject the sample. Dissolve proteins and peptides in 5% formic acid in 20–50% methanol and inject 0.5–2 µL into the nanoelectrospray needle using a micropipet with a gel loader tip.
5. Connect the needle holder to a 20-mL syringe or pressurized gas which provides the backing air pressure for the nanoelectrospray ion source.
6. Position the nanoelectrospray needle 1.5–2 mm from the orifice of the mass spectrometer. Monitor the position of the needle tip with a microscope or a video camera.
7. Gently pressurize the needle by air using the 20 mL syringe or an adjustable gas valve.
8. If no liquid appears at the needle tip then briefly and gently touch it against the interface plate of the mass spectrometer under microscopic control. The needle is not visibly shortened but a small sample droplet appears after the contact, indicating the opening of the needle tip (*see* **Fig. 1**). Note: Needle and plate should be at the same electric potential. A tiny droplet will appear on the metal plate—it spreads out as a faint shadow in a few seconds.
9. Reposition the needle in front of the orifice of the mass spectrometer.
10. Apply the voltage to needle/holder and mass spectrometer interface and start scanning the mass spectrometer. If there are ions in the spectrum reduce the air pressure in the needle to the lowest value that still keeps the flow stable.

3.2. Sample Preparation for Nanoelectrospray Mass Spectrometry

The nanoelectrospray ion source unfolds its full potential when the available sample is concentrated to 1 µL or less. Microcolumns packed in glass capillaries or in gelloader pipette tips can be used to desalt and concentrate protein and peptide samples to microliter volumes ***(3,13,14)***. A pulled glass capillary or a partially constricted gelloader tip is packed with a few hundred nanoliters of Poros resin (Applied Biosystems, Boston, MA). Working in the perfusion mode, Poros material generates only a small flow resistance when packed into a capillary. Peptide solutions are normally desalted/concentrated on Poros R2 resin, protein solutions on Poros R1 resin, and hydrophilic samples (such as small peptides and phosphopeptides) on R3 material (anion-exchange resin).

Purification of phosphopeptides or highly negatively charged peptides can be accomplished by immobilized metal affinity chromatography (IMAC) using Fe(III) or Ga(III) ions ***(15,16)***.

3.3. Sample Desalting and Concentration Employing Glass Capillary Microcolumns

For peptide analysis we routinely use the Poros R2 material (Applied Biosystems) as chromatographic resin. Remove the smallest particles by sedimenting the resin 3–5•in methanol. In a 1.5-mL microcentrifuge tube make a slurry of 10–20 µL resin in 1.2 mL of methanol.

1. Mount a pulled glass capillary into a custom-made capillary holder (Fig. 3A) or into a pierced lid of a 1.5-mL microcentrifuge tube. Use a micropipet with a gel loader tip to transfer 5 µL of resin slurry into the capillary.
2. Load the chromatographic resin into the tip of the glass capillary by centrifugal force using a manually operated tabletop minicentrifuge (e.g. PicoFuge, Stratagene, Palo Alto, CA) at low speed (500–2000 rpm). The pulled glass capillaries used for the columns are the same as used for nanoelectrospray emitters but they are not sputter-coated with metal. The chromatographic material is visible against a dark background but nearly invisible in front of white paper.
3. When sufficient chromatographic resin has been loaded into the capillary (1–2 mm resin height) the glass tip is widened/broken by gently touching it against the tabletop. The opening should allow liquid but not column resin to exit the capillary during centrifugation. Do not centrifuge the resin too fast; otherwise it becomes compressed and may block the flow of liquid. The centrifugal capillary column is only used once to avoid sample-to-sample contamination.
4. Rinse the capillary column by injecting 5 µL of 50% MeOH followed by gentle centrifugation.
5. Equilibrate the column resin by injecting 5 µL of 5% formic acid into the capillary followed by centrifugation until all liquid has passed through the column.
6. Dissolve the sample in 10–20 µL of 5% formic acid and inject it onto the capillary column in aliquots of 5 µL followed by centrifugation. For best recovery, dissolve the dried protein or peptide sample in 80% formic acid and then immediately dilute it to 5% by addition of water.
7. Wash the column resin twice by centrifugation with 5 µl of 5% formic acid solution. This desalting step is very efficient as the column is washed with 50–100• its resin volume. Before beginning the elution step the washing solution must be completely removed by gentle centrifugation.
8. Elution of sample into a nanoelectrospray needle. Mount the capillary column in-line with a premade nanoelectrospray needle in a custom-made capillary holder that fits into a microcentrifuge (Fig. 3A). Elute the peptide mixture into the nanoelectrospray capillary by centrifugating twice with 0.5 µL of 60% methanol–5% formic acid. Elute proteins with 60–70 % acetonitrile–5 % formic acid. This procedure allows handling of elution volumes between 10 µL and 0.2 µL. Elution, however, should be performed twice because the first elution does not completely

deplete the column. Keep in mind that signal intensity in an electrospray spectrum is concentration dependent, so keep the elution volume as small as possible.

9. Mount the loaded nanoelectrospray needle onto the ion source and begin the experiment.

3.4. Sample Desalting and Concentration Employing Microcolumns Packed in GELoader Tips (see Notes 1 *and* 2)

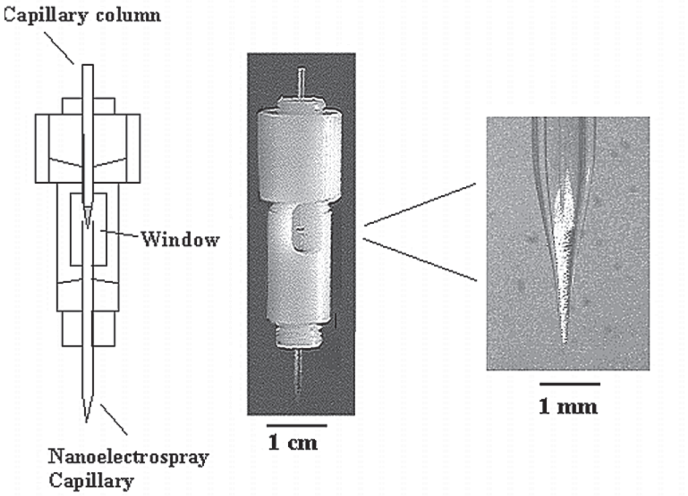
This sample desalting/concentration method is very simple and can be used prior to MALDI-MS and nanoelctrospray MS. The resin is held in place by making a constriction at the end of a GELoader pipettor tip. Sample loading, washing and elution is performed by loading liquid on top of the resin and applying air pressure to generate a low flow through the column. No frits are necessary.

1. Prepare a slurry of 100–200 µL of chromatographic resin, for example, Poros R1, R2, or OligoR3, in 1 mL of methanol.
2. Make a partially constricted GELoader pipet tip by gently squeezing or twisting the end of the tip **(Fig. 3B).** This allows liquid to flow through the tip while retaining the chromatographic resin.
3. Use another GELoader tip to load 5 µL of slurry of resin into the GELoader tip from the top and pack it by applying air pressure with the 1-µL syringe adapted to fit the GELoader tip microcolumn. The column height should be 2–4 mm.
4. Equilibrate resin by flushing 10–20 mL of 5% formic acid through the GELoader tip by air pressure (syringe). 5 Redissolve the peptide or protein sample in 20–40 mL of 5 % formic acid. 6 Load 5–20 µL of sample onto the microcolumn and gently press it through the column by air pressure using the syringe 7 Wash resin by flushing 10–20 µL of 5% formic acid through the packed GELoader tip by air pressure (syringe).
5. Elute the sample using a small volume of 5% formic acid–50% methanol. The eluate can be collected in a microcentrifuge tube, deposited directly onto the MALDI probe tip, or eluted directly into a nanoelectrospray needle. In the latter two cases the elution volume should be 1–2 µL only.

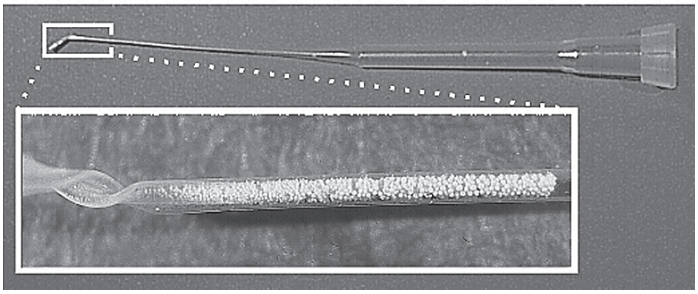
3.5. Nanoelectrospray Tandem Mass Spectrometry of Unseparated Peptide Mixtures

Peptide sequencing with tandem mass spectrometry ***(17,18)*** consists of three steps: (1) measuring the *m/z* values of peptides in a sample and determination of the charge state z; (2) acquiring the tandem mass spectra after collision-induced dissociation (i.e., fragmentation) of selected peptides; and (3) interpreting the tandem mass spectrometry data. With the nanoelectrospray source the first two steps are performed in one experiment with the unseparated peptide mixture.

The *m/z* values of analyte peptides are detected by comparing a "single MS" mass spectrum to a representative spectrum of the autolytic peptides of the enzyme used (i.e., trypsin) or a representative control from a particular experiment. For



A Nanoscale column packed in glass capillary



B Nanoscale column packed in GELoader tip

Fig. 3. Microcolumns for peptide and protein desalting/concentration prior to nanoelectrospray mass spectrometry. (**A**) Capillary column made from a pulled glass capillary and filled with a small volume of Poros resin. The sample is rinsed and then eluted from the column capillary directly into the nanoelectrospray needle by centrifugal force using a tabletop centrifuge. (**B**) Microcolumn made from a GELoader tip. The sample is loaded and eluted by air pressure from a syringe adapted to the GELoader tip.

electrophoretically isolated proteins it is often advantageous to process an empty gel piece excised near the protein band of interest as control. The charge states of peptide ions are determined by considering the isotopically resolved ion signal: An isotope spacing of 0.5 Da corresponds to a doubly charged species whereas an isotope spacing of 0.33 Da reflects a triply charged ion.

At subpicomole protein amounts on the gel it is possible to employ the precursor ion scan capability of the triple-quadrupole tandem mass spectrometer to detect peptide ion signals that were below the chemical noise in the normal Q_1 spectrum **(Fig. 4)** ***(19)***. Precursor ion scans of the abundant *m/z* 86 immonium ion of isoleucine/leucine detect peptides that contain these common amino acids. For the parent ion scan the mass spectrometer parameters are adjusted to obtain optimum detection efficiency at reduced mass resolution. The parent ion scan technique can also be used to selectively detect phosphopeptides by monitoring *m/z* 79 in the negative ion mode ***(19–21)***, to selectively detect glycopeptides by monitoring *m/z* 162 or 204 in the positive ion mode ***(19,22)***, and to detect intact proteins or oligonucleotides in contaminated samples ***(23)***. A similar method has recently been implemented on Q-TOF type instruments ***(24)***.

Once a set of peptide *m/z* values has been determined by either "single MS" scans or by precursor ion scans, high-resolution scans can be performed for selected peptide ion signals in the "multiple ion monitoring" mode to determine the exact peptide mass and the peptide charge state based on the isotope spacing. The reduction of sensitivity when measuring at high resolution is compensated by adding many scans, for example, 50 or more, to one spectrum. The latter feature further demonstrates the utility of long measurement times

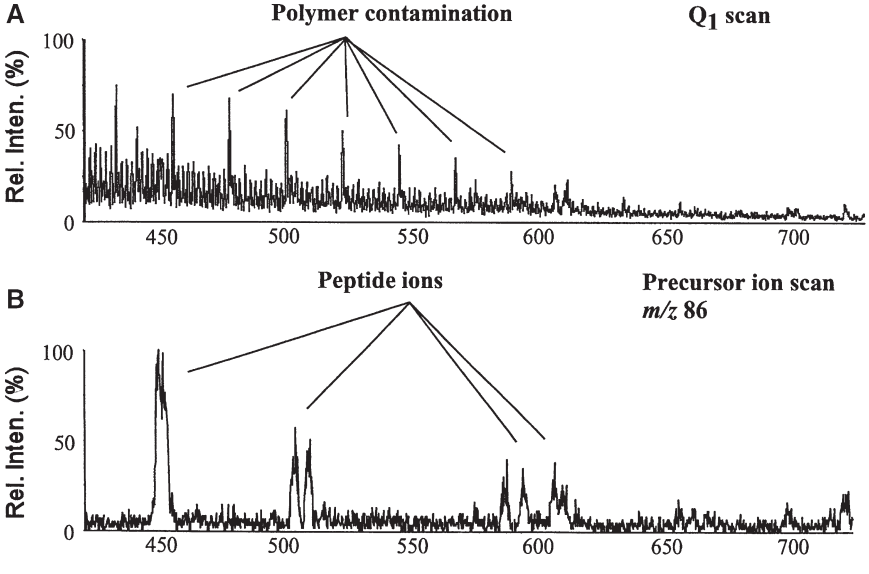


Fig. 4. Parent ion scan for the leucine/isoleucine immonium ion (*m/z* 86) detects peptide ions below the chemical noise level. (**A**) The nanoelectrospray quadrupole mass spectrum (Q1 scan) of a tryptic peptide mixture displays only polymer ion signals and chemical background which suppress peptide ions. (**B**) The nanoelectrospray triple-quadrupole MS/MS precursor ion (*m/z* 86) analysis of the same sample reveals several peptide ion signals that subsequently can be selected for sequencing by product ion analysis by tandem mass spectrometry.

that the nanoelectrospray source provides. It is advantageous to select doubly charged tryptic peptide ions for tandem mass spectrometry experiments because they generate relatively simple fragment ion spectra. Triply charged tryptic peptides can also be fragmented and often allow determination of long stretches of amino acids sequence, that is, 15–25 consecutive residues, via doubly charged fragment ion series.

3.6. Fragmenting Peptides by Collision-Induced Dissociation

Once a set of peptide *m/z* values has been accurately determined each peptide is fragmented in turn. For peptide sequencing by tandem mass spectrometry using a triple quadrupole or a Q-TOF instrument two main instrumental parameters are adjusted to obtain high quality amino acid sequence information. First, the resolution setting of the first quadrupole (Q_1) is adjusted according to the abundance of the peptide ion signal, that is, the lower the ion intensity the higher the resolution setting in order to reduce the chemical background noise in the lower half of the tandem mass spectrum for a better signal-to-noise ratio. Second, the collision energy can be adjusted according to the peptide mass and varied depending on the mass range scanned **(Fig. 5)**. The collision gas pressure is kept constant throughout the MS/MS experiment.

It may be advantageous to acquire a tandem mass spectrum in two or three segments. The high *m/z* segment is acquired with a wide parent ion selection window (low resolution) and a low collision energy to generate and detect

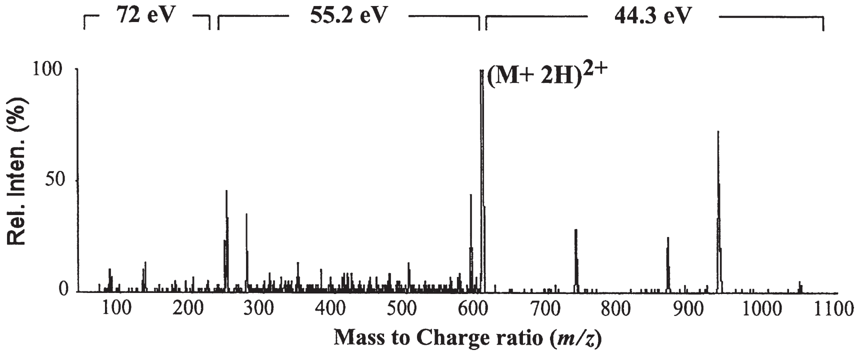


Fig. 5. Peptide tandem mass spectrum acquired in separate segments in a triple-quadrupole instrument. The *m/z* range above the precursor ion, $[M+2H]_{2+}$, is acquired at low collision energy and at relatively low mass resolution to efficiently generate and detect large peptide fragment ions. The *m/z* range below the precursor ion is acquired at higher resolution and with stepped collision energies, that is, intermediate collision energy to generate low *m/z* sequence ions and high collision energy to generate immonium ions and the a_2 and b_2 fragments.

relatively large peptide ion fragments. The low *m/z* region is acquired at higher resolution and at higher collision energies to generate and detect low *m/z* fragments and immonium ions. The nanoelectrospray allow this and other types of optimization due to the stability and long duration of the spray.

When investigating a peptide mixture by tandem mass spectrometry as many peptides as possible should be fragmented. This motivated the development of semiautomatic software routines to assist in data acquisition. The list of peptide *m/z* values is stored by customized software that calculates the optimum hardware settings for subsequent sequencing of each individual peptide. However, for *de novo* sequencing of long stretches of amino acid sequence it is not yet advisable to use automated software routines for data acquisition. Careful adjustments of collision energy and mass resolution is required to obtain high-quality data for unambiguous sequence assignments.

3.7. Generation of Peptide Sequence Tags from Tandem Mass Spectra of Peptides

Complete interpretation of tandem mass spectra of peptides can be complicated and requires some experience. However, it is often relatively straightforward to generate short consecutive sequences of two to five amino acid residues from a tandem mass spectrum. This information is valuable for sequence database searches as follows. A "peptide sequence tag" is assembled from the peptide mass, a short internal sequence of consecutive amino acid residues, and the distance in mass to the N- and C-terminus of the peptide *(25)* **(Fig. 6)**. The search specificity of this construct is very high because the amino acid sequence is "locked" in place by the masses of the "unknown" parts of the peptide. The modular composition of a peptide sequence tag makes it tolerant to errors in any one of the modules. As only a fraction of the information content of the tandem mass spectrum is used to generate a peptide sequence tag used to querythe sequence database, the remaining information confirm a retrieved peptide sequence: Every significant fragment ion signal should correlate to the peptide sequence. A peptide sequence tag consisting of three residues typically retrieve only one protein from a database containing more than 500,000 sequences when using high mass accuracy data obtained on a Q-TOF tandem mass spectrometer *(26)*. If longer stretches of sequence can be read out of a tandem mass spectrum, that is, six or more residues, it is advantageous to search by amino acid sequence instead of by peptide sequence tags. Searching by amino acid sequence is more flexible and allows sequence homology searches.

As mentioned previously, tryptic peptides have the desirable feature that they contain an N-terminal amino group and a C-terminal Lys or Arg residue, localizing protons at both the N-terminus and the C-terminus of the peptide. Tandem mass spectra of tryptic peptides very often contain a continuous y-ion series that can

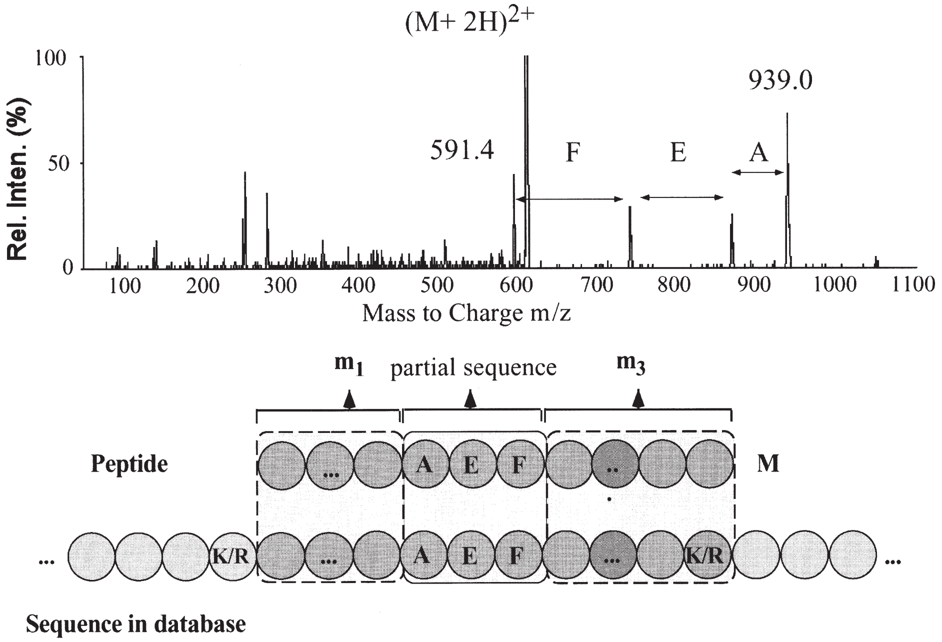


Fig. 6. eptide sequence tag generated from a tandem mass spectrum. A short search string, (591.4)FEA(939.0), is readily identified in the tandem mass spectrum of this tryptic peptide with mass M. It is subsequently converted to a peptide sequence tag, e.g. by using the Mascot software (www.matrixscience.com) and used to query a database. The modular composition of a peptide sequence tag permits error tolerant searches where one or two of the modules are allowed to contain an error.

be readily assigned in the *m/z* range above the doubly charged parent ion signal. The spectrum interpretation strategy builds on this characteristic. It is guided by the demand to identify a protein in sequence databases or to reliably sequence peptides for cloning of the cognate protein.

Several algorithms can identify proteins based on sequence database searches with uninterpreted peptide tandem mass spectra ***(27,28).*** Such software tools are very useful for a first screening of peptide tandem mass spectra because they immediately identify peptides originating from known proteins. The Mascot search engine and a number of other useful Internet-based services can be found via the URL http:// www.protein.sdu.dk.

3.8. Guidelines for Interpreting Tandem Mass Spectra of Tryptic Peptides

The following list summarizes a few basic empirical rules that we use in interpreting tandem mass spectra of tryptic peptides. Because peptides differ in their fragmentation behavior in a sequence-dependent manner it is possible to find exceptions to these rules.

1. The goal of the interpretation is to find a series of peaks that belong to one ion series—for tryptic peptides mostly y-ions (C-terminal fragments).
2. Initial peak selection: The high *m/z* region of a tandem mass spectrum is often straightforward to interpret. Choose a large ion signal in this region as the "starting peak."
3. Assembly of a partial amino acid sequence: Try to find ion signals that are precisely one amino acid residue mass away from the starting peak (up or down in mass). We use software which marks all the possibilities in the spectrum. This provides a good overview whether there is more than one possibility for sequence assignments. If there is a repeating pattern of fragment ion peaks with satellite peaks representing an H_2O loss (−18 Da) or an NH_3 loss (−17 Da) a fragment ion series has been identified (for tryptic peptides a y–ion series is more likely).
4. By repeating step 3 a peptide sequence tag consisting of two to four amino acids is assembled that is subsequently used to identify a protein in the sequence database. As default for tryptic peptides, the database is searched under the assumption that a y–ion series was determined. However, even for tryptic peptides the tandem mass spectrum can be dominated by b-ions if a peptide contained an internal basic residue or when the C-terminal peptide of the protein had been sequenced.

3.9. Confirming Protein Identifications Madevia Peptide Tandem Mass Spectra

If a protein sequence is retrieved by a database search with a peptide sequence tag or by similar methods then the amino acid sequence of the retrieved peptide should fit the tandem mass spectrum in order to be called as a positive match. Two or more peptides from a sample should independently identify the same protein in a database. To verify a match, the peptide fragment masses must be correct within the expected error of the mass measurement. For tryptic peptides the y-ion series should be nearly complete, except when a peptide contains an internal proline residue (*see below*). The N-terminal b_2 and a_2 fragment ions, generated at relatively high collision energy, should be present in the low *m/z* region. Odd fragmentation patterns should reflect the amino acid sequence as discussed in the following paragraphs.

Peptides that contain internal basic residues (lysine or arginine) do not fragment in the vicinity of these residues because a charge is localized at the sidechain of the basic residue and therefore not available for amide backbone cleavage. If the triply charged precursor ion was fragmented then doubly charged y ions are present in the spectrum.

Internal proline residues deserve special attention. Cleavage of the C-terminal bond of a proline is observed to a low degree. The N-terminal bond of a proline is labile giving rise to an intense y–ion fragment. Internal fragmentation of peptides containing a proline often confirm a sequence: The y–ion generated by fragmentation at the N-terminal side of Pro will dissociate a second time to produce a y–ion series which is superimposed on the original y–ion series. However, the b–ions generated from this Pro containing fragment serve to confirm the C-terminal part of a peptide sequence up to and including the internal proline residue.

Isoleucine and leucine cannot be differentiated by amide bond cleavage alone because they have the same elemental composition and therefore identical molecular weight. Pairs of amino acid with identical nominal mass, Lys/Gln (128 Da) and oxidized Met/Phe (147 Da), can often be distinguished. Lys and Gln differ in their basicity and trypsin cleaves C-terminal to Lys and not at Gln so internal Lys is rarely found in tryptic peptides. However, if the latter is the case then the tryptic peptide usually acquire an additional proton for a total of three charges and the tandem mass spectrum will often contain a doubly charged y-ion series. If an internal Lys residue is suspected then the peptide mixture can be inspected for the presence of the limit peptide produced by tryptic cleavage at this Lys residue. Oxidized methionine (147.02 Da) and phenylalanine (147.07 Da) residues are differentiated relatively easily as follows. Tandem mass spectra of peptides which contain an oxidized methionine residue (i.e., methionine sulfoxide) display satellite peaks that appear 64 Da below each methionine sulfoxide-containing y-ion fragment due to loss of CH_3SOH from methionine sulfoxide ***(29,30)***. The oxidation reaction is often not complete so inspection of the peptide mass spectrum (Q_1 spectrum) may reveal a peptide 16 Da below the one containing oxidized methionine. The high resolution and mass accuracy of Q-TOF, LTQ-FT-ICR and LTQ-FT-Orbitrap type MS instruments enable differentiation of Gln/Lys and OxMet/Phe by accurate mass determination.

If no proteins are retrieved with a simple database search then redo the search under the assumption that some of the amino acids in the peptide are modified, containing for example, an oxidized methionine or a *S*-acrylamidocysteine. An error tolerant search can be launched in which only partial correspondence between the peptide sequence tag and a database entry is required ***(25)***. Additional information about the protein can be used to select possible candidates if more than one protein sequence is retrieved (such as protein size, organism, or function).

If a protein cannot be identified by any of its peptide sequence tags we conclude that it is unknown. For proteins from human, mouse, or other model organisms, the peptide sequence tags are then screened against a database of expressed sequence tags (ESTs) ***(31)***. ESTs are short stretches of cDNA, that is, single-stranded DNA generated from expressed mRNA by reverse transcriptase, and thus represent the set of expressed genes in a given cell line. If a database search retrieves a cDNA sequence then library screening and cloning is straightforward. If the EST database search produces no hit then *de novo* peptide sequencing has to be pursued to obtain longer stretches of amino acid sequences for homology searches. It has been demonstrated that peptide MS/MS data is useful to identify genes in raw genomic sequences *(32)*.

3.10. De Novo Peptide Sequencing by Nanoelectrospray Tandem Mass Spectrometry

To sequence unambiguously an unknown protein for homology searching and cloning, it is necessary to be able to confidently call extended series of b-type and y-type fragment ion signals.

A useful method to recognize y-ion series employes ^{18}O-labeled water (*see*, e.g., **refs.** ***26, 33, 34***). By performing the trypsin digestion in a 1:1 mixture of normal water and ^{18}O labeled water all the tryptic peptides will incorporate ^{18}O at the C-terminus with a yield of approx 50%. Each peptide ion appears in a mass spectrum as a doublet separated by 2 Da. Selecting both isotope species together for fragmentation (low resolution setting in Q_1 precursor ion selection) produce tandem mass spectra that display y–ions as a series of split peaks, that is, separated by 2 Da, whereas b–ions are single peaks. This isotope-splitting is easily resolved in modern tandem mass spectrometers, particularly Q-TOF instruments ***(26)*** and aids in the interpretation of a peptide tandem mass spectrum because y-ion series are confidently assigned **(Fig. 7)**. Note that the ^{18}O-labeled

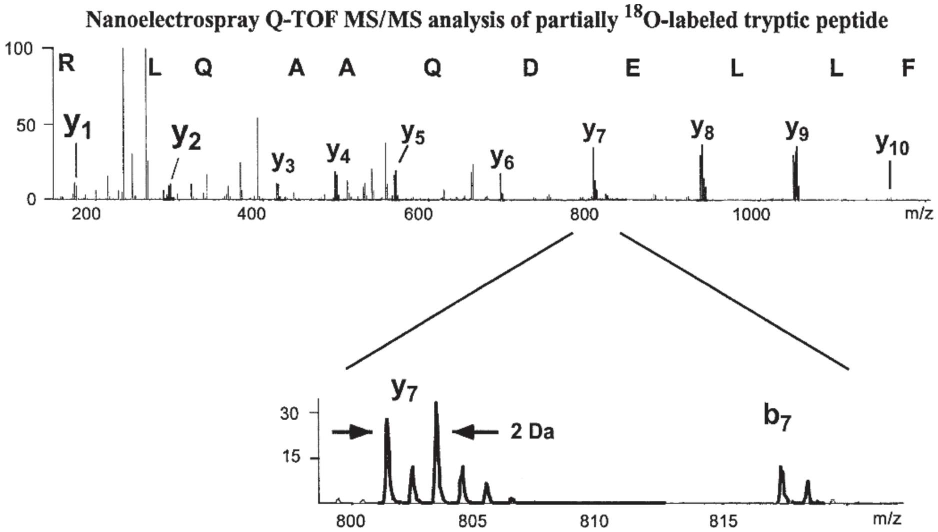


Fig. 7. Incorporation of the O stable isotope in peptides facilitates sequence interpretation in MS/MS. Digestion of protein by trypsin a solution of $H_2^{16}O$/ $H_2^{18}O$ water (1:1) results in partial labeling of the C-terminal carboxyl group in each peptide, except the C-terminal peptide of the protein. Identification of y-ion series is now straightforward due to appearance of y-ion signal peak splitting in a 1:1 ratio and a mass spacing of 2 Da.

water has to be very pure to avoid chemical background noise. Redistillation of commercially available ^{18}O-labeled water is recommended.

Another method uses derivatization of free carboxyl groups, including the C-terminal carboxyl group of peptides. The tryptic peptide mixture is split in two portions. The first portion of the mixture is analyzed by nanoelectrospray tandem mass spectrometry and long peptide sequences are generated through complete interpretation of tandem mass spectra using the guidelines described in the preceding. The other portion of the peptide mixture is *O*-methyl-esterified ***(17,35,36)*** and then analyzed. Every free carboxyl-group including the C-terminus of peptides is esterified and therefore increase in mass by 14 Da. The number of methyl-esters in a peptide can be determined by the mass shift of peptides which is predictable from the previously interpreted tandem mass spectra of the native peptides. Because all y–ion fragments produced from an esterified peptide contain the C-terminus they are all shifted up in mass. Comparison of a set of tandem mass spectra obtained from a peptide and the corresponding esterified peptide serve to confirm the amino acid sequence because the y–ion series can unambiguosly be assigned. In addition, internal acidic residues, Asp and Glu, are methylated as well and can easily be differentiated from their corresponding amide residues, Asn and Gln, which otherwise differ in mass by only 1 Da.

3.11. Perspectives

Novel peptide sample preparation methods, mass analyzer configurations, and peptide dissociation techniques are continuously developed to increase sensitivity, mass accuracy, mass resolution, or sample throughput for peptide mass analysis and sequencing by mass spectrometry. The combination of the MALDI source with a Q-TOF hybrid instrument ***(37)*** and the development of electron capture dissociation (ECD) and electron transfer dissociation (ETD) for MS/MS sequencing of large peptides and small intact proteins ***(38)*** are just two recent examples. Posttranslational modification of proteins leads to a change in the molecular mass of the affected residues and mass spectrometry is, therefore, a versatile analytical tool for structural characterization of modified peptides and proteins ***(39,40)***. MS-based approaches to peptide and protein quantitation using stable isotope labeling are also being pursued ***(41)*** and the use of multidimensional chromatography methods combined with ESI-MS/MS is an alternative or complement to two-dimensional gel electrophoresis for analysis of very complex protein mixtures ***(42–44)***. There is no doubt that applications of mass spectrometry in biological research will expand substantially in the future as the structure and function of all the gene products encoded in genomes of model organisms, including humans, have to be characterized in molecular details.

4. Notes

1. Peptide separation may be improved by eluting the sample from the microcolumn by a step gradient using a series of mobile phases containing 5% formic acid in 15% methanol, 30% methanol, and 50% methanol, respectively.
2. For MALDI-MS analysis, elute the sample directly onto the MALDI probe using matrix solution, for example, HCCA, SA, or 2,5-dihydroxybutyrate (2,5-DHB) dissolved in 30–50% methanol or acetonitrile. Deposit the eluate in a series of tiny droplets rather than one large drop ***(13)***. Trifluoroacetic acid or acetic acid can be used instead of formic acid in the mobile phases, whereas acetonitrile can substitute for methanol. Formic acid and methanol are recommended for nanoelectrospray mass spectrometry.
3. Troubleshooting: The nanoelectrospray ion source is a very robust device. However, problems may occur if the sample contains high concentrations of buffers, polymers or salts or if the shape of the needle tip is not within the dimensions described in the preceding. In this subheading we provide a few troubleshooting tips.

   a. If there are no ions or no noise in the spectrum then the needle is not spraying. Repeat step 8 in Protocol 1 to open the needle tip.
   b. If the spray becomes instable then jitter and spikes will appear in the spectrum or the spectrum will contain an unusually high level of chemical background. Increasing the air pressure of the needle helps stabilize the flow. If this simple measure is insufficient then the spraying voltage may be too low (increase it by 100–150 V) or the opening of the needle tip is still too small (repeat step 8 in **Subheading 3.1**).
   c. Be aware that the nanoelectrospray needle has a very small diameter. Small changes in the applied potential (increases of 100 V) change the field density at the tip considerably. Electrical discharge can be initiated and ionize atmospheric gas which generates a mass spectrum. These ions are usually small (<400 Da), and therefore chemical background ions in the higher mass region are missing. Atmospheric gas ionization can be visible as a blue corona around the needle tip (when light sources are switched off) and may lead to oxidations of methionine containing peptides ***(12)*.**
   d. If opening of a needle is not successful by the means described in the preceding then apply voltage to the needle, pressurize it, and briefly touch it against the mass spectrometer interface plate (the potential difference between needle and interface plate should be about 500 V). The combined mechanical and electrical stress opens almost every needle. It is not advisable to routinely open needles by this approach because

it tends to damage the metal coating at the tip. High electrical current drawn from very thin tips can heat the glass to a degree that the glass melts. The damaged piece of the tip can often be broken off and analysis can proceed with a larger opening.

e. Two effects can prevent or stop spraying from an opened needle: The high surface tension of the liquid or precipitation of salts, polymers, or other nonvolatile sample constituents when their concentration is high. In the former case the needle can easily be reopened by slightly touching it to the interface plate, thereby destroying the surface tension by physical contact. In the latter case, the harsher procedure employing mechanical and electrical stress for needle opening (described earlier) can be attempted.

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Protein Identification by Peptide Mass Fingerprinting using MALDI-TOF Mass Spectrometry

Judith Webster and David Oxley

1. Introduction

Developments in mass spectrometry technology, together with the availability of extensive DNA and protein sequence databases and software tools for data mining, has made possible rapid and sensitive mass spectrometry-based procedures for protein identification. Two basic types of mass spectrometers are commonly used for this purpose; MALDI-TOF-MS and ESI-MS. MALDI-TOF instruments are now quite common in biochemistry laboratories and are very simple to use, requiring no special training. ESI instruments, usually coupled to capillary/nanoLC systems, are more complex and require expert operators. We will therefore focus on the use of MALDI-TOF-MS, although the sample preparation is identical for both methods. The principle behind the use of MALDI-TOF-MS for protein identification is that the digestion of a protein with a specific protease will generate a mixture of peptides unique to that protein. Measuring the molecular masses of these peptides then gives a characteristic dataset called a peptide mass fingerprint (PMF) *(1)*. The PMF data can then be compared with theoretical peptide molecular masses that would be generated by using the same protease to digest each protein in the sequence database, to find the best match. Provided the protein being analyzed is present in the database being searched and the data is of sufficient quality, the best match should be the correct protein. In order to judge the validity of a protein identification by this method, some means of scoring the quality of the match must be used.

The procedure described here involves cutting protein bands or spots from 1-D or 2-D PAGE gels, destaining the gel pieces, reducing and alkylating the

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protein, digesting with trypsin, using MALDI-TOF mass spectrometry to determine the mass of the tryptic peptides and database searching with the PMF data to identify the protein.

The sample processing steps can be performed in microfuge tubes or in 96 well plates. One person can easily process a 96 well plate in a day, but if higher throughput is required, each step can be automated, allowing the possibility of several hundred protein identifications per day. Spot cutting, sample processing and sample plate loading robots are commercially available and routinely used in many high-throughput laboratories.

2. Materials

1. 0.5 mL microfuge tubes (or 96 well V-bottom polypropylene microtitre plates) (*see* **Note 1**).
2. One Touch Spot Picker (1.5 mm) (The Gel Company) - optional.
3. Silver destaining solution (for silver stained gels only) - dissolve potassium ferricyanide (2 mg/mL) in sodium thiosulphate solution (0.2 mg/mL). Make fresh immediately before use.
4. Sonicator bath (or vortex mixer with attachments for unattended use with 0.5 mL microfuge tubes or 96 well microtitre plates).
5. Aqueous buffer: 50 m*M* NH_4HCO_3 – make fresh weekly.
6. Organic buffer: 50 m*M* NH_4HCO_3/acetonitrile 1:1 – make fresh weekly.
7. DTT solution: (DL-Dithiothreitol ultra pure) 10mM DTT in aqueous buffer - make fresh immediately before use.
8. Iodoacetamide solution: (ultrapure) 50 m*M* iodoacetamide in aqueous buffer - make fresh immediately before use. Note iodoacetamide is toxic!
9. Trypsin solution (Modified Sequencing Grade, Promega) (*see* **Note 2**).
10. Centrifugal vacuum concentrator.
11. Matrix solution (*see* **Note 3**).
12. Peptide calibration mixture (*see* **Note 3**).
13. MALDI sample plate.
14. MALDI-TOF mass spectrometer.

3. Methods

Protein identification by PMF is usually performed on protein bands or spots cut from 1-D or 2-D PAGE gels and so this is the method described here, however it can also be applied to proteins in solution with minor modifications. One of the biggest problems that may be encountered, particularly when low amounts of protein are analyzed, is contamination. **Note 1** describes some precautions to minimize the effects of contamination.

All washing steps during the processing of the gel pieces can be done either on a vortex mixer or in a sonicator bath.

3.1. Removing Gel Spot or Band from the Gel and Destaining

Ideally, the gel should have been stained with a Coomassie stain (preferably a colloidal Coomassie if detection sensitivity is an issue, (e.g. ***(2, 3)*** or one of the commercially available stains). This method is also compatible with Sypro stains (though a uv transilluminator will be required to visualize the protein bands or spots during excision). Standard silver stains are not compatible, but certain modified silver stains (e.g. ***(4),*** or one of the commercially available "mass spectrometry compatible" silver stains) can be used, however, even these usually give inferior mass spectrometry data compared to Coomassie or Sypro stained gels.

3.1.1. Removal of Gel Piece

1. Place the stained gel in a disposable petri dish on a light box and cut out the spot/band with a manual spot picker or a clean scalpel/razor blade, without taking any excess gel.
2. If necessary, cut the gel spot/band into 1mm pieces and transfer into a 0.5mL microfuge tube or one well of a 96 well microtitre plate.

3.1.2. Destaining Coomassie or Sypro-Stained Gel Pieces

1. Wash with aqueous buffer (100 µL) 1 × 5 min.
2. Wash with organic buffer (100 µL) 2 × 5 min.

3.1.3. Destaining Silver-Stained Gel Pieces (5).

1. Wash with silver destaining solution (100 µL) until destained (approx 5 – 30min) - the gel will retain a pale yellow colour.
2. Wash with high purity water (100 µL) 2 × 5 min.
3. Wash with aqueous buffer (100 µL) 1 × 5 min.
4. Wash with organic buffer (100 µL) 1 × 5 min.

3.2. In-Gel Reduction and Alkylation of the Protein

Reduction of disulphide bonds followed by alkylation of the free cysteines to prevent reoxidation, while not essential for the digestion of most proteins, generally gives better results. This is due to increased susceptibility of the reduced/alkylated protein to tryptic digestion and the absence of any disulphide-linked peptides (which are not matched in the database search) from the PMF data ***(6).***

1. Incubate destained gel pieces in DTT solution (100 µL) for 1h at 50°C in an oven. If using a 96 well plate, use sealing film or a sealing lid, to exclude oxygen and prevent drying out.
2. Cool to room temperature, remove and discard DTT solution. Add iodoacetamide solution (100 µL) and incubate with occasional mixing (vortex) for 1 h in the dark at room temperature.

3. Discard supernatant and wash gel pieces with aqueous buffer (100 µL) for 5 min, then with organic buffer (100 µL) for 2 × 5min.
4. Dry the gel pieces completely in a centrifugal vacuum concentrator. Caution should be exercised in handling the dried gel pieces, as they are easily lost from tubes or plates.

3.3. In-Gel Digestion of the Protein with Trypsin

1. Take an aliquot of trypsin (10 µL of 10× stock solution) from the freezer, add aqueous buffer (90 µL) and mix.
2. Rehydrate gel pieces for 10 min in trypsin solution (approx. 1 µL per mm^3 gel). There should be little or no excess liquid after rehydration.
3. Add an equal volume of aqueous buffer and incubate at 37°C overnight (or for at least 3 h). Use an incubator, not a heating block. If using a 96 well plate, use sealing film or a sealing lid, to prevent drying out.
4. Add 1/10 th volume of 10% TFA and sonicate/vortex for 5 min.
5. The resulting supernatant is used directly for MALDI-TOF-MS.
6. Residual peptides can be washed from the gel piece if required with a small amount of 0.1% TFA.

3.4. Mass Determination of Peptides by MALDI-TOF-MS

The quality of the MALDI-TOF spectrum that will be obtained from the sample depends crucially on the sample/matrix preparation. The basic requirements are for a uniform microcrystalline layer of matrix/sample and the removal of salts and other contaminants. There are numerous published methods, but the one described here is robust and quite simple, requiring no preclean-up steps. For the best results, a high quality matrix, as supplied by MALDI-MS manufacturers or in a commercial PMF kit, should be used. Alternatively, analytical grade matrix can be recrystallized *(7)*.

MALDI-TOF spectra must be calibrated in order to achieve sufficient accuracy for database searching. This is done by acquiring spectra on peptide standards to generate a calibration curve, which is applied to the experimental data. The calibration peptides can be analyzed separately from the experimental sample (external calibration) or they can be mixed with the experimental sample (internal calibration). Internal calibration is more accurate (typically 10–20 ppm for a strong spectrum) than external (typically 100 ppm or more), but is more difficult as the amount of standard peptides used needs to be matched to the level of the experimental sample peptides. The addition of too much peptide standards can suppress the signal of the sample peptides and *vice versa*. A commonly used variation of the internal calibration method utilizes the autodigestion fragments of trypsin (842.5094 and 2211.1040 for porcine trypsin) for calibration, instead of adding additional peptides *(7)*.

3.4.1. Applying the Sample and the Calibration Mixture to the MALDI Plate

1. Apply matrix solution (0.5 µL) to a clean MALDI plate and allow to dry (sample spot).
2. Apply protein digest (0.2–2 µL) to the sample spot and allow to dry. If a large amount of gel was used for the digestion, the supernatant volume could be much larger than 2 µL (up to 20 µL). It is not usually necessary to use all of the supernatant unless the protein band was quite weak. In this case the entire supernatant can be concentrated to 1–2 µL and used (*see* **Note 4**).
3. Apply 0.1% TFA (5–10 µL) to each sample spot, leave for 30 s, remove and discard, then repeat; this step desalts the sample.
4. If using external calibration, apply 0.2 µL of peptide calibration mixture as close as possible (but not touching) to each sample spot and allow to dry (calibrant spot).

3.4.2. Measuring the Peptides Masses in the MALDI-TOF-MS Instrument

The precise operation of the MS is instrument-dependent, but the basics are very similar. Increasing the laser power increases the signal, but also decreases the resolution and "burns" off the sample faster. Therefore, the laser power should be set to the lowest level that gives a good signal. Set the mass range (e.g. m/z 600–3500), the number of shots to be acquired (100–200 is reasonable) and set the laser power low. Gradually increase the power until an even distribution of noise appears across the whole mass range. Within a few shots, peaks should begin to appear above the noise. Continue to adjust the laser power until the signal level is satisfactory.

The heterogeneous nature of the matrix surface means that some areas of the sample spot will give better peptide signals than others. The difference can be dramatic, so it is a good idea to periodically move the laser position, while assessing the signal intensity, to find the hotspots.

If external calibration of the spectrum is to be used, a spectrum of the calibration standards should be acquired immediately, using the same power setting. **Fig. 1** shows a typical MALDI spectrum obtained from the trypsin digestion of a weak colloidal Coomassie-stained 1D gel band.

3.5. Generating PMF Data and Searching Protein Databases

The PMF data must now be extracted from the MALDI spectrum using the appropriate software associated with the MS instrument used. The baseline threshold should first be adjusted to ensure that all of the peptide signals are detected, without including any noise. If the spectrum has been obtained on a high resolution (reflectron) instrument, the peptide signals will appear as multiple peaks separated by 1Da, due to the presence of ^{13}C isotopes in the peptides

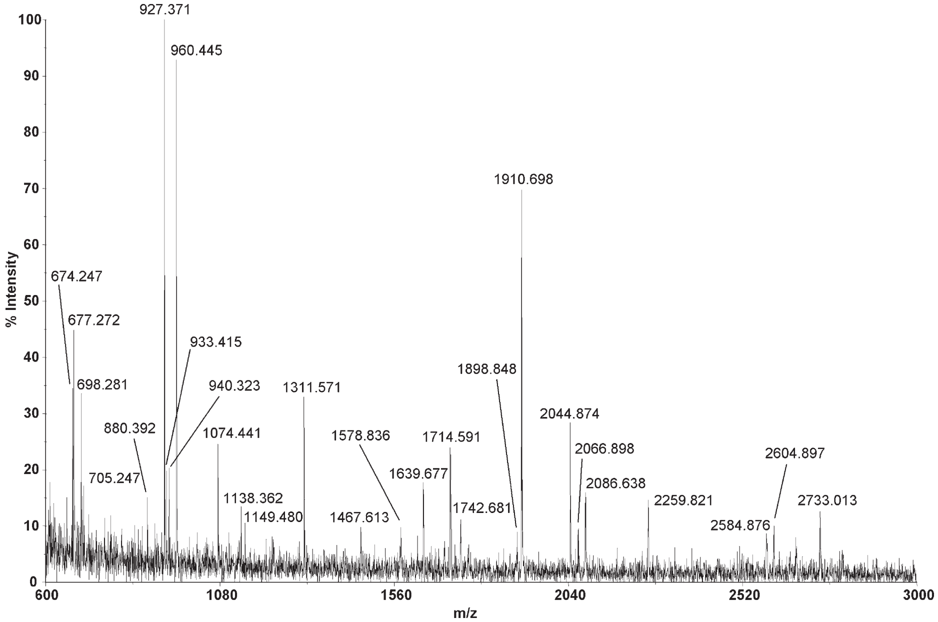


Fig. 1. MALDI-TOF spectrum of a trypsin digested 1-D gel band. Peaks are labelled with their monoisotopic masses. Note that these are not the masses of the peptides, but of the peptide (pseudo)molecular ions. In MALDI spectra, peptide molecular ions arise predominantly through the addition of a proton to the peptide, giving a mass increase of 1.007 Da. The molecular ions are usually denoted as MH+ or [M+H]+.

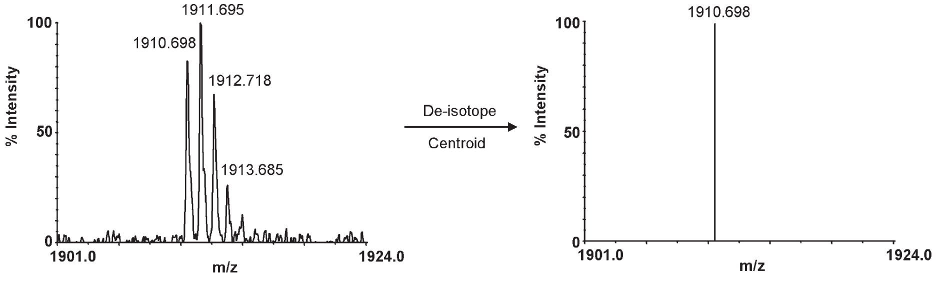
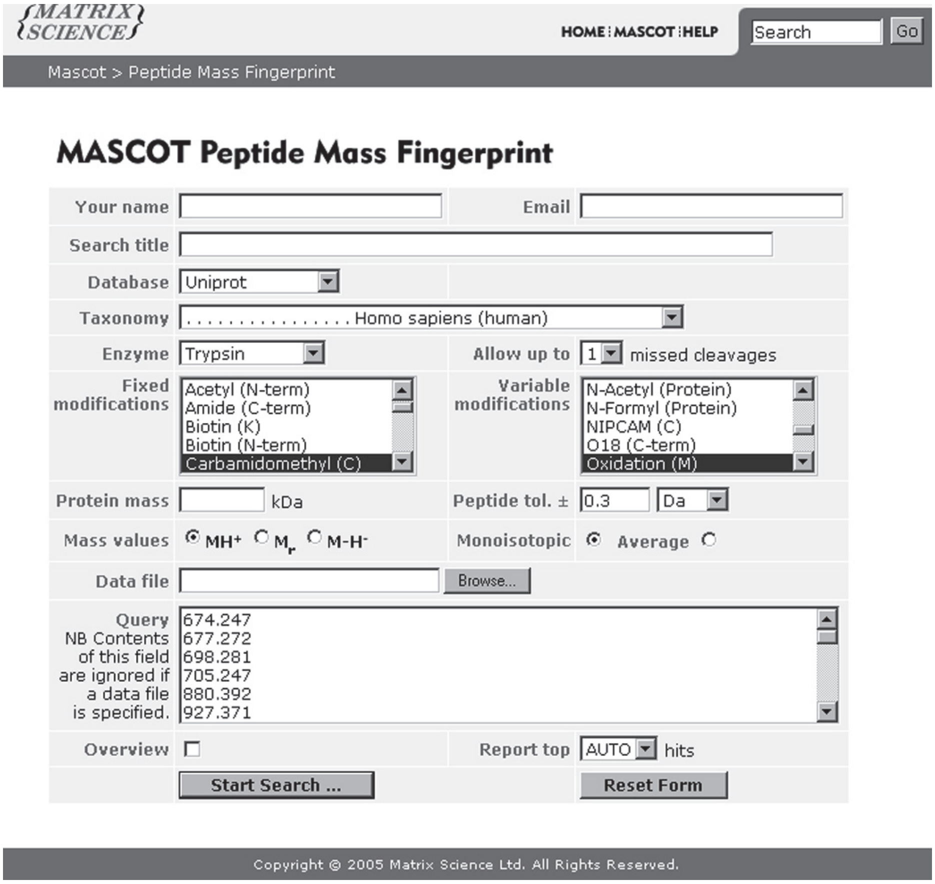


Fig. 2. Expanded view of the peptide signal at around m/z 1910 from the MALDI-TOF spectrum in **Fig 1**. De-isotoping removes the ^{13}C isotope peaks and centroiding reduces the remaining peak to a single data point.

(*see* **Fig. 2**). "De-isotoping" and "centroiding" of the MS data is necessary, so that only the monoisotopic peptide masses are included in the PMF data. The MS data analysis software will have an option for the data to be shown as a list of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MASCOT Peptide Mass Fingerprint

Your name
Email
Search title
Database: Uniprot
Taxonomy: . . . . Homo sapiens (human)
Enzyme: Trypsin
Allow up to: 1 missed cleavages
Fixed modifications: Acetyl (N-term), Amide (C-term), Biotin (K), Biotin (N-term), Carbamidomethyl (C)
Variable modifications: N-Acetyl (Protein), N-Formyl (Protein), NIPCAM (C), O18 (C-term), Oxidation (M)
Protein mass: kDa
Peptide tol. ±: 0.3 Da
Mass values: ● MH+ ○ M_r ○ M-H-
Monoisotopic ● Average ○
Data file
Query NB Contents of this field are ignored if a data file is specified.:
674.247
677.272
698.281
705.247
880.392
927.371
Overview ☐
Report top: AUTO hits

Fig. 3. Mascot PMF search page.

masses (peak list), which can be saved as a text file or simply copied directly into the data entry field of the database search engine. There are a number of search engines that can be used for PMF searches, some of which are freely available on the internet (*see* **Note 5**). We will use Mascot to demonstrate a search, but all have essentially the same functions.

The Mascot PMF search page is shown in **Fig. 3**. Detailed explanations of the various terms and parameters are available from the web site; brief descriptions are given in **Note 6**.

1. Select the database to be searched from the drop-down list e.g. Uniprot.
2. If taxonomic information is known for the sample origin, select from the list, otherwise select “All entries”.
3. Select “trypsin” as the enzyme.
4. “Allow up to” 1 missed cleavage.

5. Select “Carbamidomethyl (C)” as a fixed modification.
6. Select “Oxidation (M)” as a variable modification.
7. If the MALDI spectrum was internally calibrated, the peptide tolerance can be set to 0.1 Da, otherwise 0.3 to 0.5 Da is a reasonable starting point.
8. Select “MH+” for Mass values.
9. Select “Monoisotopic” if the MALDI data were acquired in reflectron mode, or “Average” for data acquired in linear mode.
10. If the peak list was saved as a text file, browse to the file location, otherwise paste the list directly into the “Query” field.
11. Select “Auto” from “Report top” hits.
12. Start search.

Within a few seconds the search result will appear which should look similar to **Fig. 4**. This shows a graphical representation of the results and a list of significant matches. A single significant match is indicated by the graph with a maximum score of 226, well above the significance threshold of 61. The score is related to the probability that the match is real rather than purely random, and is also expressed as an expect value equivalent to the BLAST E-value, 1.8e-18 in this case, indicating that this is almost certainly a genuine match.

In the “Concise Protein Summary Report” shown, all of the proteins matching the same set or subset of masses are listed under a single entry. Typically, these will represent multiple database entries for the same protein as well as sequence variants, fragments, and so on.

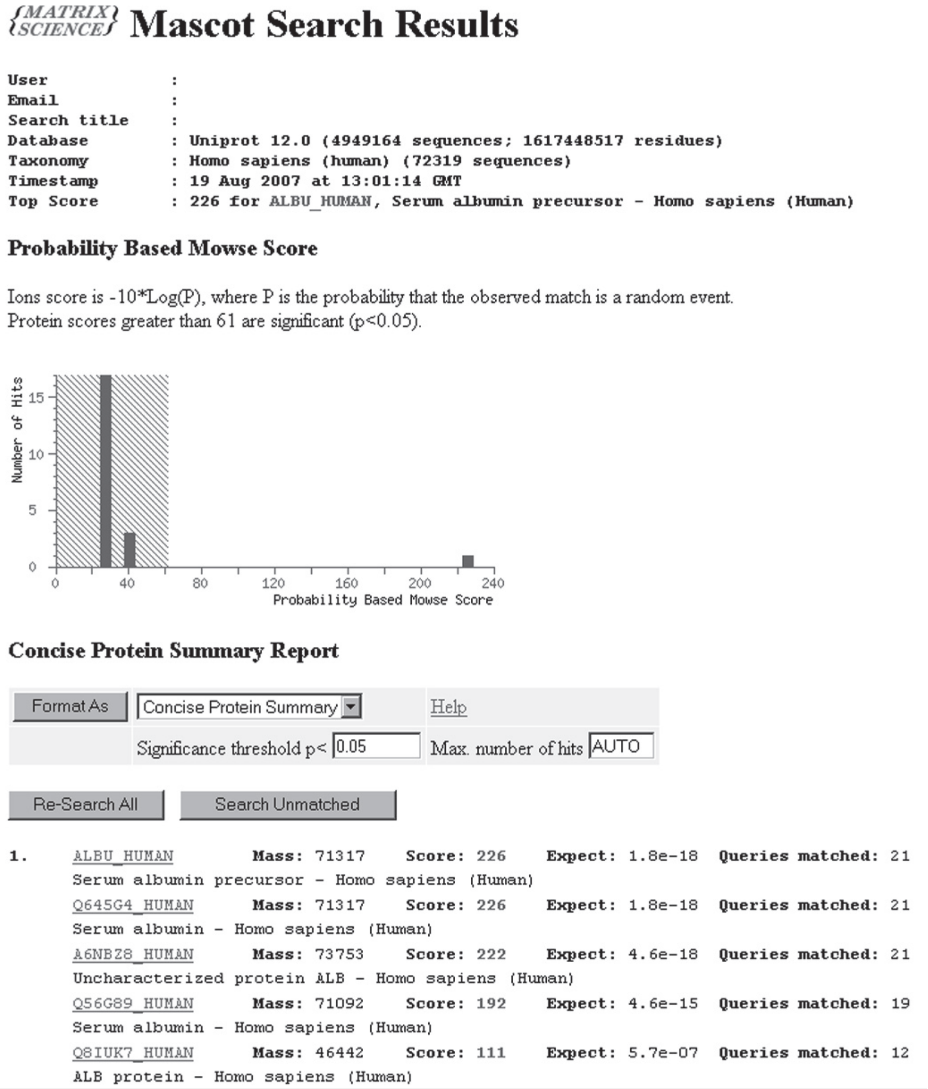
Clicking on a protein accession number from the list brings up further information (*see* **Fig. 5**), including the matched peptides, listed and mapped onto the protein sequence, as well as the % sequence coverage and any unmatched masses. There will almost inevitably be some unmatched masses; they can be due to the presence of contaminating proteins in the digest, non-tryptic cleavages, incomplete digestion, modified peptides, errors in the database sequence etc ***(8).***

The graph at the bottom of **Fig. 5** plots the error for each of the matched peptide masses and is useful for assessing whether the tolerance setting used for the search was appropriate. If desired, a more appropriate setting could be chosen and the data researched.

3.6. Failure to Obtain a Significant Hit

Some of the most common reasons why a database search may not give a significant match. are:

1. Too few masses in the PMF data – depending on the size of the database and the parameters settings, a minimum of 4–6 matched peptides are required for a significant hit. This situation could arise simply because the spectrum is very weak and so only a few of the strongest peptide signals are visible, or because the particular protein does not yield many peptides within the useful mass range (e.g. small



Mascot Search Results

```
User          :
Email         :
Search title  :
Database      : Uniprot 12.0 (4949164 sequences; 1617448517 residues)
Taxonomy      : Homo sapiens (human) (72319 sequences)
Timestamp     : 19 Aug 2007 at 13:01:14 GMT
Top Score     : 226 for ALBU_HUMAN, Serum albumin precursor - Homo sapiens (Human)
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 61 are significant (p<0.05).

Concise Protein Summary Report

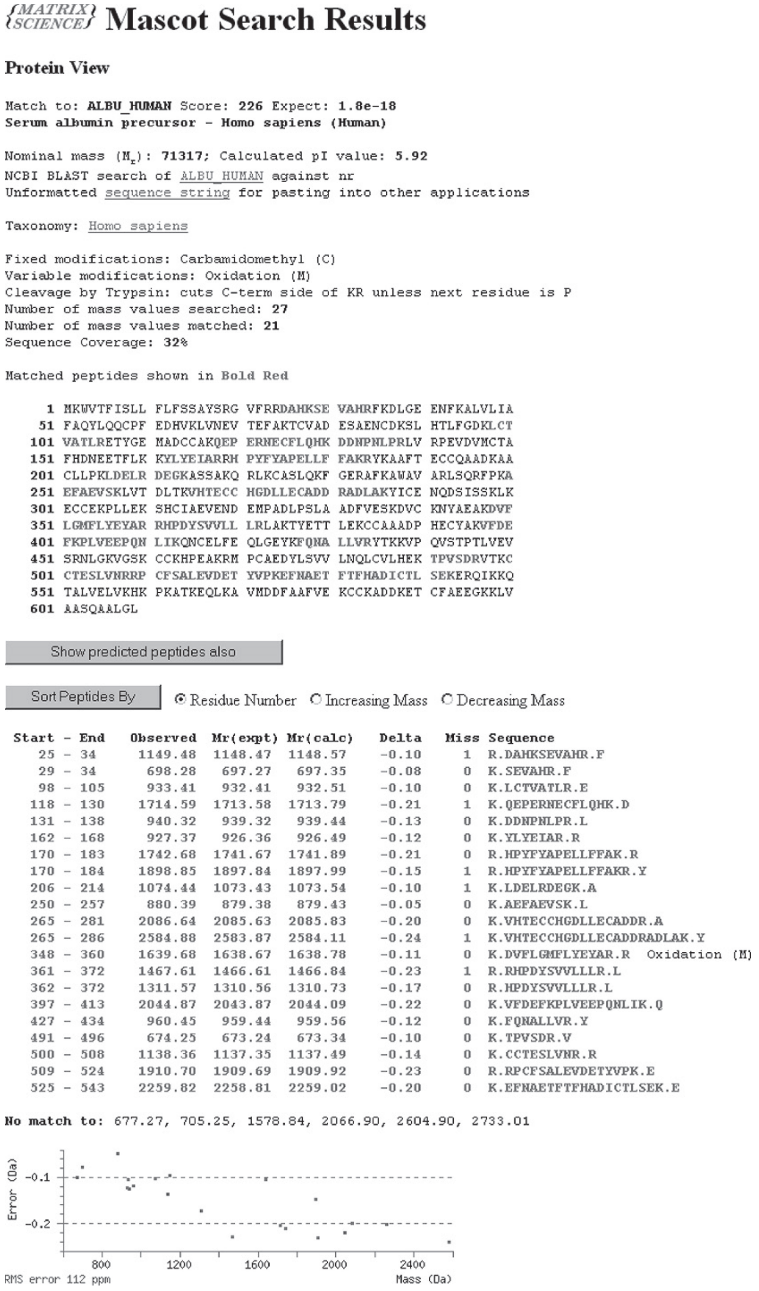
Significance threshold p< 0.05 Max. number of hits AUTO

```
1.   ALBU_HUMAN      Mass: 71317   Score: 226   Expect: 1.8e-18   Queries matched: 21
     Serum albumin precursor - Homo sapiens (Human)
     Q645G4_HUMAN    Mass: 71317   Score: 226   Expect: 1.8e-18   Queries matched: 21
     Serum albumin - Homo sapiens (Human)
     A6NBZ8_HUMAN    Mass: 73753   Score: 222   Expect: 4.6e-18   Queries matched: 21
     Uncharacterized protein ALB - Homo sapiens (Human)
     Q56G89_HUMAN    Mass: 71092   Score: 192   Expect: 4.6e-15   Queries matched: 19
     Serum albumin - Homo sapiens (Human)
     Q8IUK7_HUMAN    Mass: 46442   Score: 111   Expect: 5.7e-07   Queries matched: 12
     ALB protein - Homo sapiens (Human)
```

Fig. 4. Mascot search results summary page using PMF data from the spectrum in **Fig 1.**

proteins, proteins with very high or very low numbers of potential trypsin cleavage sites, proteins which are resistant to trypsin digestion).

2. Mass spectral noise included in the PMF data – ensure that the peak detection threshold is not set too low.
3. More than one protein present in the sample – with good quality PMF data, mixtures containing 2–3 proteins can be identified, but the presence of peptide masses from the other proteins reduces the score for each individual match. With lower quality



Mascot Search Results

Protein View

```
Match to: ALBU_HUMAN Score: 226 Expect: 1.8e-18
Serum albumin precursor - Homo sapiens (Human)

Nominal mass (Mr): 71317; Calculated pI value: 5.92
NCBI BLAST search of ALBU_HUMAN against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 27
Number of mass values matched: 21
Sequence Coverage: 32%

Matched peptides shown in Bold Red

    1 MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA
   51 FAQYLQQCPF EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT
  101 VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV RPEVDVMCTA
  151 FHDNEETFLK KYLYEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA
  201 CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV ARLSQRFPKA
  251 EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
  301 ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF
  351 LGMFLYEYAR RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE
  401 FKPLVEEPQN LIKQNCELFE QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV
  451 SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV LNQLCVLHEK TPVSDRVTKC
  501 CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ
  551 TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
  601 AASQAALGL
```

● Residue Number ○ Increasing Mass ○ Decreasing Mass

```
 Start - End    Observed  Mr(expt) Mr(calc)    Delta   Miss Sequence
    25 - 34      1149.48  1148.47  1148.57    -0.10     1  R.DAHKSEVAHR.F
    29 - 34       698.28   697.27   697.35    -0.08     0  K.SEVAHR.F
    98 - 105      933.41   932.41   932.51    -0.10     0  K.LCTVATLR.E
   118 - 130     1714.59  1713.58  1713.79    -0.21     1  K.QEPERNECFLQHK.D
   131 - 138      940.32   939.32   939.44    -0.13     0  K.DDNPNLPR.L
   162 - 168      927.37   926.36   926.49    -0.12     0  K.YLYEIAR.R
   170 - 183     1742.68  1741.67  1741.89    -0.21     0  R.HPYFYAPELLFFAK.R
   170 - 184     1898.85  1897.84  1897.99    -0.15     1  R.HPYFYAPELLFFAKR.Y
   206 - 214     1074.44  1073.43  1073.54    -0.10     1  K.LDELRDEGK.A
   250 - 257      880.39   879.38   879.43    -0.05     0  K.AEFAEVSK.L
   265 - 281     2086.64  2085.63  2085.83    -0.20     0  K.VHTECCHGDLLECADDR.A
   265 - 286     2584.88  2583.87  2584.11    -0.24     1  K.VHTECCHGDLLECADDRADLAK.Y
   348 - 360     1639.68  1638.67  1638.78    -0.11     0  K.DVFLGMFLYEYAR.R  Oxidation (M)
   361 - 372     1467.61  1466.61  1466.84    -0.23     1  R.RHPDYSVVLLLR.L
   362 - 372     1311.57  1310.56  1310.73    -0.17     0  R.HPDYSVVLLLR.L
   397 - 413     2044.87  2043.87  2044.09    -0.22     0  K.VFDEFKPLVEEPQNLIK.Q
   427 - 434      960.45   959.44   959.56    -0.12     0  K.FQNALLVR.Y
   491 - 496      674.25   673.24   673.34    -0.10     0  K.TPVSDR.V
   500 - 508     1138.36  1137.35  1137.49    -0.14     0  K.CCTESLVNR.R
   509 - 524     1910.70  1909.69  1909.92    -0.23     0  R.RPCFSALEVDETYVPK.E
   525 - 543     2259.82  2258.81  2259.02    -0.20     0  K.EFNAETFTFHADICTLSEK.E

No match to: 677.27, 705.25, 1578.84, 2066.90, 2604.90, 2733.01
```

Fig. 5. Detailed information for the protein identified in the Mascot search results in **Fig. 4**.

data, this can result in all of the scores dropping below the significance threshold. The presence of contaminants e.g. trypsin or keratins has the same effect, however, known peptide masses derived from keratins and trypsin can be removed from the PMF data prior to searching ***(9).***

4. Peptide tolerance set too low or too high.
5. Incorrectly calibrated spectrum.
6. Searching EST databases – ESTs are generally short and therefore do not usually provide sufficient numbers of matched peptides for a significant score.
7. The protein is not present in the database being searched.

4. Notes

1. Contamination is a serious concern when attempting to identify low amounts of protein by MALDI-MS. Use only the highest quality reagents, HPLC grade solvents and high quality plasticware to minimize non-protein contamination. Ideally a set of glass/plasticware and reagents/buffers should be dedicated for MALDI-MS analysis. Laboratory dust is a major source of protein contaminants (keratins) and so every effort to exclude dust from samples, buffers and reagents must be taken. Communal buffers, stains, destains, and so on, are often contaminated with dust and should be avoided. Rinse all glassware with high quality water before making up buffers etc. Wear nitrile gloves (latex contains protein contaminants) and lab a coat. Keep a cover over the gel during staining, and so on. Never stain a gel in a container that has been used for processing Western blots, as they will be contaminated with blocking proteins. Only handle the gel if absolutely necessary and then avoid touching parts of the gel that are to be cut. Gel pieces can be cut and processed in laminar flow cabinets, although this is usually not necessary if sensible precautions are taken.
2. Modified trypsin is preferred for protein digestion as it is less susceptible to autodigestion ***(7).*** The trypsin 10x stock solution is prepared by dissolving the trypsin (20μg) in the solvent supplied with the enzyme (50 m*M* acetic acid; 200 μL). This is stored at −70°C in aliquots (10 μL) and is stable for at least one year.
3. MALDI analysis kits (e.g. Sequazyme peptide mass standards kit from Applied Biosystems) are a convenient way to obtain matrix, peptide standards and solvents, however these items can easily be purchased independently. Any peptides which span a reasonable proportion of the useful mass range can be used for calibration, provided their accurate molecular weights are known.

   α-cyano-4-hydroxycinnamic acid is the most common matrix used for peptide analysis. Matrix solution is prepared by dissolving 5mg of matrix in 1 mL of 4:1 acetonitrile/water containing 0.1% TFA.

   A mixture of peptide calibration standards e.g. des-Arg^1-bradykinin (monoisotopic mass 904.4681), Angiotensin 1 (monoisotopic mass

1,296.6853), ACTH (1–17 clip) (monoisotopic mass 2,093.0867), ACTH (18–39 clip) (monoisotopic mass 2,465.1989) is made to a concentration of 2 pmol/µL each in 0.1% TFA in water. This solution is then mixed 1:1 with matrix solution to give the final peptide calibration mixture.

4. Larger volumes of digests can be concentrated by vacuum centrifugation, but this can lead to significant peptide losses, particularly if the sample is concentrated to dryness. Alternatively, peptides can be concentrated and desalted by using a micro scale pipette-tip format solid-phase extraction device (e.g. micro C18 Zip Tips from Millipore, Omix C18MB tips from Varian or STAGE tips from Proxeon). The details of their use in desalting peptide solutions for MALDI analysis are given with the product, but for the final elution step, use the matrix solution described above, to elute the peptides directly onto the MALDI plate.
5. Freely available PMF search engines include: Mascot from Matrix Science (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF), MS-Fitfrom Protein Prospector (http://prospector.ucsf.edu/cgi-bin/msform.cgi?form=msfitstandard) and Aldente from ExPaSy (http://www.expasy.org/tools/aldente).
6. SwissProt is a relatively small, but highly annotated database with minimal redundancy. Uniprot, NCBInr and MSDB are much larger, but have multiple entries for many proteins. Other search engines may have additional database choices and in-house copies allow custom databases to be used.

   Specifying the taxonomy as closely as possible reduces the number of database entries which need to be considered in the search. This reduces search times, but more importantly, reduces the threshold score for significant matches, increasing the confidence of any protein identification.

   The cleavage specificity of trypsin is C-terminal to Lys and Arg residues (except where followed by Pro). However, not every such peptide bond will be cleaved. The number of missed cleavages to consider can be specified, but increasing the number decreases the significance of any match and should normally be set to 1.

   Fixed modifications are modifications to specific amino acids which are considered to be complete i.e. every occurrence of the amino acid in the sequence is assumed to carry the modification and the unmodified amino acid is not considered. Variable modifications, on the other hand, are incomplete and therefore both the modified and unmodified amino acid is considered in the search. In the example discussed above, the reduction/alkylation should result in complete carbamidomethylation of all cysteine residues, thus "Carbamidomethyl (C)" was chosen as a fixed modification, whereas methionine oxidation, a common artifactual modification which is usually incomplete, was selected as a variable modification. The use of multiple variable modifications will greatly reduce the significance of any match and should therefore be used with caution.

Most PMF search engines allow a molecular weight for the protein to be entered; the search will then consider only database entries within a window around this value. This can be dangerous, as proteins are subject to processing/degradation which can significantly increase or decrease their molecular weight. Mascot uses a more complex method of applying this parameter, but in most cases this can be left blank.

The peptide tolerance is a window around each mass value in the peak list within which a theoretical database peptide mass must fall, in order to be matched.

Acknowledgments

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Protein Ladder Sequencing

Rong Wang and Brian T. Chait

1. Introduction

The ladder peptides are generated by stepwise chemical degradation in a controlled manner. In our first experiment, we obtained ladders by a manual method using stepwise Edman degradation in the presence of a small amount of terminating agent ***(1)***. Subsequently, three additional chemical approaches have been developed for generating N-terminal peptide ladders. The first of these employed short coupling reaction times (incomplete reaction) in an automatic gas-phase amino acid sequencer ***(2)***, while the second used volatile coupling reagents in a set of manual reactions ***(3)***, and the third utilized rapid acid hydrolysis reaction with microwave irradiation ***(4)***. In meanwhile, new terminating agents of Edman degradation were developed and tested for producing N-terminal peptide ladders, such as allyl isothiocyanate (AITC) ***(5)***. More recently, a new terminating agent, N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSU), was reported ***(6)***. Following the Edman degredation, the Fmoc group can be removed from the N-terminus and any reacted side chains by piperidine treatment and yield N-terminal peptide ladders without chemical modifications ***(6)***. Although we are discussing peptide ladder generation by chemical means, the peptide ladders can also be produced by enzymatic digestions using exopeptidase, such aminopeptidase and carboxypeptidase. The application of carboxypeptidases is the major experimental method to generate C-terminal peptide ladders for protein ladder sequencing ***(7–11)***.

Here, we will describe our early manual approach, using phenylisocyanate (PIC) as the terminating agent to produce the latter peptides ***(1,12)***. A small quantity of 5% phenylisocyanate (PIC) is used as the terminating agent in the coupling step. The resulting phenylcarbamyl (PC) peptide derivatives are stable to

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the trifluoroacetic acid (TFA) used in the subsequent cleavage step. A small fraction of N-terminally blocked peptide is generated at each cycle. A predetermined number of cycles are performed without intermediate separation or analysis of the release amino acid derivatives. The resulting mixture of peptides (the ladder) is measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry ***(13)***. The mass spectrum contains peaks corresponding to each terminated peptide species present. The amino acid residues are identified from the mass differences between consecutive peaks. The order of these mass differences in the data set gives the amino acid sequence of the original peptide **(Fig. 1)**.

Protein ladder sequencing has several potential advantages compared with conventional amino acid sequencing.

1. The amino acid sequence is read out from one spectrum. Thus, optimally, all members of the sequence-defining fragments of the peptide, each differing by one

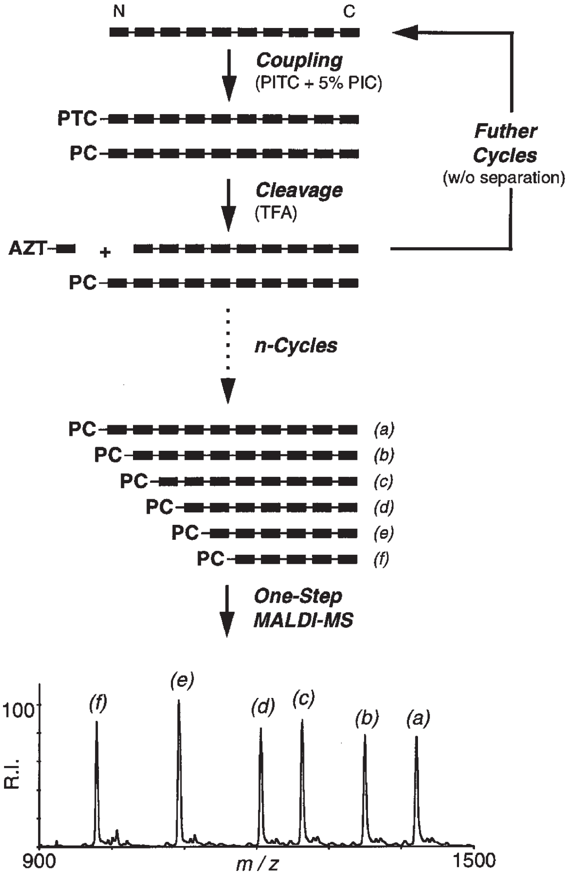

Fig. 1. The principle of N-terminal protein ladder sequencing. A stepwise Edman degradation is carried out in the presence of small of amount of terminating reagent (phenylisocyanate, PIC) in the coupling step. The resulting phenylcarbomyl (PC) peptides are stable to trifluoroacetic acid (TFA) used to cleave the terminal ammo acid from the phenylthiocarbomyl (PTC) peptides in the cleavage step. A ladder peptide mixture is formed as the result of PC-peptide accumulation in successive Edman cycles. Each black block represents an amino acid residue.

amino acid residue, are simultaneously examined. The sequence of the peptide is deduced from the set of fragmentation products. Such a data set contains mutually interdependent information that determines the identity and the order of each amino acid residues in the parent peptide. Carry over resulting from incomplete sequencing reactions cause no ambiguities in the present method.

2. The method can be used to obtain sequence information from peptide mixtures.
3. Direct detection of posttranslational modifications can be made with ladder sequencing ***(14,15)***.
4. The ladder generating chemistry does not require complicated apparatus and can be done in any chemistry laboratory. However, the current procedure requires at least 100 pmol of peptide, since sample loss generally occurs in the liquid-phase extraction.

Protein ladder sequencing has the potential to be used in the following biological applications.

1. In conjunction with peptide fragment mass mapping, the method can provide additional sequence information and facilitate protein sequence database searching for protein identification ***(8)***.
2. The method can be used to study the nature and to determine the sites of chemical modifications and posttranslational modifications (e.g., phosphorylation, and glycosylation) ***(14,15)***.
3. In protein processing and metabolism pathway studies, the method can be used to identify and confirm the terminal of protein processing products ***(16).***

2. Materials

2.1. Chemicals and Reagents (see Note 1)

1. Phenylisothiocyanate (PITC) [Thermo Scientific (Pierce), Rockford, IL or Sigma-Aldrich Co., St. Louis, MO].
2. Phenylisocyanate (PIC), >98% (Sigma-Aldrich Co.).
3. Pyridine [Thermo Scientific (Pierce)].
4. Hexafluoroisopropanol (HFIP, 99.8+% (Sigma-Aldrich Co.).
5. Heptane [Applied Biosystems Inc., Foster City, CA or Thermo Scientific (Pierce)]
6. Ethyl acetate [Applied Biosystems Inc., Thermo Scientific (Pierce), or Sigma-Aldrich Co.].
7. Trifluoroacetic acid (TFA), anhydrous [Thermo Scientific (Pierce)].
8. 12.5% Trimethylamine (TMA) (Applied Biosystems Inc.).
9. α-Cyano-4-hydroxycinnamic acid, 97% (Sigma-Aldrich Co.).
10. Acetonitrile (Applied Biosystems Inc.).
11. Nitrogen, 99.99% (Matheson, Montgomeryville, PA).
12. Distilled and deionized water (prepared by using Milli-Q UV Plus water purification system).

2.2. Laboratory Equipment

1. Eppendorf Micro-Centrifuge (Brinkmann Instruments, Westbury, NY).

2. Savant SC110 Speed-Vac (Savant Instruments, Farmingdale, NY).
3. Multi-Block Heater (Lab-Line Instruments, Melrose Park, IL).
4. Fisher Vortex (Fisher Scientifics, Pittsburg, PA).
5. In-house laboratory vacuum.
6. Polypropylene microcentrifuge tube (PGC Scientifics, Gaithersburg, MD).

2.3. Mass Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (*see* **Note 2**).

2.4. Preparing Reagents (see Note 3)

1. Coupling buffer: Pyridine/water (1:1, v/v, pH 10.1). 250 µL water and 250 µL pyridine.
2. Coupling reagent: PITC/PIC/pyridine/HFIP (24:1:72:3, v/v). (Prepared under a blanket of dry nitrogen). 120 µL PITC, 5 µL PIC, 360 µL pyridine, 15 µL HFIP.
3. Cleavage reagent: 500 µL TFA.
4. Conversion buffer: 12.5% TMA/pyridine (1:1, v/v). 100 µL 12.5% TMA, 100 µL pyridine.
5. Conversion reagent: PIC/pyridine/HFIP (1:80:2.5, v/v). 2 µL of PIC, 160 µL pyridine, 5 µL HFIP.
6. Washing solvent A: Heptane/ethyl acetate (10:1, v/v). 10 mL heptane, 1 mL ethyl acetate.
7. Washing solvent B: Heptane/ethyl acetate (2:1, v/v). 8 mL heptane, 4 mL ethyl acetate.
8. Mass spectrometric matrix solution. 2 mg α-cyano-4-hydroxycinnamic acid, 200 µL of 0.1% TFA in water, 100 µL of acetonitrile. Vortex vial thoroughly to suspend the solid into liquid phase to form turbid mixture and then centrifuge the turbid mixture at 16,000g for 2 min. The saturated matrix solution will be used in the sample preparation for mass spectrometric analysis.

3. Methods

3.1. Ladder Generating Reaction by PITC/PIC – Wet Chemistry

This is manual method to produce ladder peptides based on a manual Edman chemistry ***(17)*** (*see* **Note 4**). All of the reactions are carried out in a 0.6-mL polypropylene microcentrifuge tube. Since PITC is very sensitive to oxidation, some of the operations are perform under a blanket of day nitrogen. Operations requiring a nitrogen blanket will be indicated.

3.1.1. Coupling Reaction

1. Dissolve peptide sample in 20 µL of coupling buffer.
2. Add 20 µL of coupling reagent (under a blanket of dry nitrogen).
3. Incubate the reaction vial at 50°C for 3 min with a block heater.

3.1.2. Removal of the Coupling/Terminating Reagents and Byproducts by Two-Phase Liquid Extraction (*see* **Note 5**)

1. Remove the reaction vial from the block heater.
2. Wipe the outside of the reaction vial with a pre-wet paper towel to condense the reaction liquid.
3. Centrifuge the reaction vial briefly at 16,000 g.
4. Add 200 µL of washing solvent A to the reaction vial, vortex gently, and centrifuge at 14,000 rpm for 1 min to clear the phase.
5. Aspirate the upper phase by vacuum suction. Extreme care should be taken to avoid loss of the lower phase liquid.
6. Repeat steps 4 and 5 one more times with washing solvent A and twice with washing solvent B. It is advisable to use a fine bore pipette tip for this task.
7. After these four extractions, dry the remaining solution in a Speed-vac centrifuge for about 5–8 min.

3.1.3. Cleavage Reaction

After the reaction mixture is dried, add 20 µL of anhydrous TFA to the reaction vial (under a blanket of dry nitrogen). Incubate the reaction vial at 50°C for 2 min with a block heater.

3.1.4. Removal of Cleavage Reagent

Remove the cleavage reagent, TFA, by drying the reaction vial in a Speed-vac centrifuge for about 5 min.

3.1.5. Conversion of Free N-Termini to Phenylcarbamyl Derivatives

After the last cycle of coupling-washing-cleavage steps, subject the total peptide mixture to an additional treatment with PIC to convert any remaining unblocked peptides to their phenylcarbamyl derivatives.

1. Add 20 µL of conversion buffer and 20 µL of conversion reagent to the reaction vial.
2. Carry out the reaction at 50°C for 5 min.
3. Extract the reagents as described in Section 3.1.2.
4. Repeat steps 1–3 one more time (*see* **Note 6**).

3.1.6. Acidify the Peptide Mixture (*see* **Note 7**)

Add 5 µL of TFA to each reaction vial and vortex briefly.

Remove TFA by a Speed-vac centrifuge for about 5 min.

3.2. Mass Spectrometric Analysis

Add 3 µL of matrix solution to the reaction vial and vortex briefly. Apply 1 µL of this peptide/matrix solution on the sample probe and dry at ambient temperature. Acquire mass spectra in the positive ion mode using a laser desorption/ionization time-of-flight mass spectrometer.

3.3. Sequencing Data Interpretation

A successful measurement will result in a mass spectrum (*see* **Note 8**) that contains a group of peaks as illustrated in **Fig. 2**. The mass spectrum is plotted using relative intensity (*y*-axis) vs mass-to-charge ratio (*x*-axis). The measured value is the ratio of mass/charge (m/z) for each ion detected by the mass spectrometer. Since the peaks observed in the spectra usually represent single protonated peptide ions (unity charge, $[M+1]^+$) in matrix-assisted laser desorption/ionization mass spectrometry, the amino acid sequence is determined by calculating the m/z or mass differences between two peaks sequentially from high mass to low mass in the mass spectrum. In the example shown in **Fig. 2**, peak (a) has an m/z value of 1419.6 Dalton and peak (b) has an m/z value of 1348.5 Dalton. The mass difference is calculated as 1419.6 – 1348.5 = 71.1 Dalton. The identity of this amino acid residue (Ala) is determined by comparing the calculated mass difference with the amino acid residue masses. The identities of other amino acid residues are determined in the same fashion as Ser (86.8), Gly (57.2), Ile/Leu (113.0), and Ile/Leu (113.2) (*see* **Note 9**). The amino acid sequence (amino-terminal to carboxy-terminal) is determined from the order of the residues identified from high to low mass of the sequencing ladder (i.e., Ala-Ser-Gly-Ile/Leu-Ile/Leu).

Chemically modified amino acid residues can be identified by the same procedure using the mass differences obtained from the spectrum *(15)* (*see* **Note 10**). These residue masses can be used to determine the identity of the modifications.

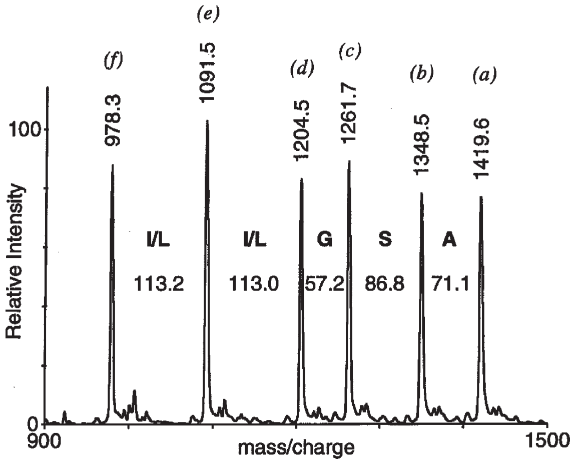

Fig. 2. Amino acid sequence read-out from a mass spectrum of ladder peptides. The vertical labels are measured m/z values for the peaks in the spectrum. The horizontal numbers are calculated mass differences between adjacent peaks. The identified ammo acid residues are indicated. The ammo-terminal sequence is read from right to left.

4. Notes

1. All chemical and solvents used in the ladder-generating chemistry should be protein sequencing grade or the highest grade available.
2. The laser desorption/ionization time-of-flight mass spectrometer was constructed at The Rockefeller University and described in detail elsewhere ***(18).*** This mstrument is equipped with a 2-m-long flight tube and uses a Nd-YAG laser source (HY 400, Lumonics, Kanata, Ontario, Canada) that generates pulsed laser light (wavelength 355 nm) with a duration of 10 ns. Commercial matrix-assisted laser desorption/ionization time-of-flight mass spectrometers should also be suitable for reading out the masses of the ladder peptides.
3. Amount of sequencing reagents prepared here are enough for sequencing four peptide samples and five cycles per sample. Fresh PITC and TFA (both in ampule) are used for each experiment.
4. A detailed discussion on manual Edman chemistry is given in ***ref. 17.***
5. Special care should be taken to remove the coupling reagents and byproducts of the coupling reaction. Otherwise, nonvolatile salt will form and affect subsequent reactions and mass spectrometric analysis.
6. It is necessary to perform the conversion twice, since the concentration of PIC is limited by its solubility and may be too low to yield 100% conversion.
7. The matrix-assisted laser desorption/ionization mass spectrometric analysis of protein and peptide requires acidic conditions. Unless the pH is lowered, the base used in the coupling reaction will significantly reduce the sensitivity of the mass spectrometric measurement.
8. It is important to note that the mass spectrometric response of a peptide is dependent on its amino acid composition. The sensitivity, in general, is enhanced when the peptide contains basic amino acid residues. On the other hand, the sensitivity is reduced when no basic amino acid residues are present. The modification on ε-amino group of lysine residues by phenylisocyanate may decrease the mass spectrometric response, especially for peptides that do not contain arginine residues.
9. Leucine and isoleucine have identical residue masses (113 Dalton). These isobaric amino acid residues cannot be distinguished by the present ladder sequencing method. Lysine and glutamine have the same nominal residue masses (128 Dalton). However, they can be distinguished from each other; since the ε-amino group of lysyl residue is modified by PIC and results in a mass shift of 119 (residue mass of PC-lysine is 247.3 Dalton).
10. To detect a posttranslational modification using ladder sequencing, the modification should not be labile to the ladder sequencing chemistry. In certain cases, ambiguous results may result from posttranslationally modified amino acid residues that have similar masses to nature amino acid residues.

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Sequence Analysis with WinGene/WinPep

Lars Hennig

1. Introduction

Retrieving information from sequence data is becoming increasingly important. Currently, a multitude of public services is available via the internet. However, many scientists are annoyed by slow network connections and busy serves. Moreover, identifying appropriate Internet pages while struggling with outdated links is often extremely time consuming. For these reasons, PC-based stand-alone applications constitute a useful alternative to Internet services. As available commercial software is usually quite expensive, WinGene and WinPep were developed that may be used free of charge by researchers in academic institutions *(1)*.

WinGene and WinPep merge diverse analysis possibilities with maximal comfort for the user. Thus, the programs combine data analysis and data presentation. Simple, general principles of Windows® programs allow immediate usage without lengthy training. Specialized analysis tasks for which Internet connections are extremely advantageous, such as structure predictions and database searches, were not included in the software. The first version of WinPep was released in 1998 *(1)* and became very popular. The latest versions, WinPep 3.01 and WinGene 2.31, contain several improvements and new functions. **Tables 1** and **2** summarize the abilities of WinGene 2.31 and WinPep 3.01, respectively. The following sections describe some features in more detail.

2. Materials

2.1. Hardware

The programs run on any PC currently marketed, but also on most older models. The computer should be at least a PC/486 equipped with 8MB RAM. WinGene/WinPep require one of the following operating systems: Windows®95,

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Table 1
Features of WinGene 2.31

	Ref.
Reverse-complement sequences	-
Restriction analyses	*(2)*
Calculation of composition and melting temperature of oligonucleotides	*(3,4)*
In-silico translation and identification of open reading frames	-

Windows®98, Windows®NT, Windows®2000, Windows®XP or Windows®Vista. At least 5MB hard disk space must be available. Connection to the internet is only needed for downloading and updating the software.

2.2. Software

WinGene/WinPep can be downloaded free of charge from http://www.pb.ethz.ch/downloads. WinGene performs restriction analysis of nucleotide sequences (cf. section 3.2.2.) requiring an enzyme database. Recent versions be obtained from REBASE (ftp.neb.com, http://rebase.neb.com) *(2)*.

3. Methods

3.1. General features

The programs come with extensive help files that explain most procedures and contain major references. Most parameters of WinGene/WinPep are saved in INI files and can be modified from within the program. Alternatively, direct editing of the INI files with any text processor is possible.

Sequence data can be entered by different means: Direct typing, *Copy&Paste*, via the clipboard, import from text files or files in FASTA format are possible (cf. **Note 1**). Moreover, WinGene allows to in silico translate nucleotide sequences into amino acid sequences and to export them directly into WinPep. All results can be easily printed or copied to the clipboard. After pasting in other applications further editing of text and graphics is possible. Moreover, graphical representations can be saved to a disk in Enhanced Windows Metafile (EMF) format. Sequences can be exported in FASTA format.

3.2. WinGene

3.2.1. Miscellaneous

With WinGene, nucleotide sequences can be easily reverse-complemented by clicking on the “Complement the sequence” button. After selecting the part of the sequence, the “Primer check” function calculates base composition and melting temperature of the selected oligonucleotides ***(3,4)***. If there was no selection, the dialog box will open for *de novo* entry of the oligonucleotide sequence. The resulting report can be printed or pasted into any text processor software, for instance for keeping records of available oligonucleotides.

3.2.2. Restriction Analysis

WinGene can be used to perform restriction analyses of given nucleotide sequences. The source file for restriction enzymes is gcg.009 from the REBASE database ***(2)*** (cf. **Note 2**). In the "Restriction Analysis" Dialog, the options for the current task can be set. The user can choose one of the following enzyme subgroups: (1) all enzymes, (2) only enzymes with recognition sites equal to or longer than a defined value, (3) a pre-selected, saved subset of enzymes ("YFE," your favorite enzymes), or (4) an arbitrary selection from the list of all enzymes. To generate an YFE list, first select the desired enzymes. Then click on "Make YFE." The file YFE01.txt will be created in the program directory. This file contains the ID numbers of the selected enzymes for fast and convenient access during subsequent analyses (cf. Note 3). In addition, the selected subset can be restricted further by activating "Only commercially available enzymes". The provided sequence will be considered as linear unless the "Circular DNA" item is checked.

Output may contain the sequence with indicated restriction sites ("Map") and/or a table of all occurrences of the recognition sequences in the input sequence ("Table"). If desired, recognition sites can be marked automatically by bold letters.

Table 2
Features of WinPep 3.01

	Ref.
Determination of amino acid composition	-
Estimation of isoelectric point	***(6)***
Calculation of molecular weight	***(6)***
Calculations of molar absorbance coefficients	***(8,9)***
Batch analyses of multiple sequences	-
Display of helical wheels	***(6)***
Display of hydropathy plots	***(5,7)***
Display of amphypathy plots	***(1)***
Search for sequence motifs	-
Simulation of site-specific cleavage	-
Identification of post-translational modifications	-
Display of domain structure	-

3.2.3. Translation and Identification of Open Reading Frames

Translation of the nucleotide sequence is initiated by clicking on the "Translate the Sequence" button. The translate window displays the sequence with its translation in three frames below. Basic text formatting (bold, underline, italics) is available. WinGene will indicate which sequence (original or complementary) is used and corresponds to the sense (upper) strand. The individual strands and frames can be de-selected from current display. This feature is useful, if for instance only display of the sense strand with one translated frame is desired. A list of all ORFs (Open Reading Frames) longer than a specified minimum (cf. Note 4) is available with the position of the first occurring methionine given. If only one frame is displayed, the longest ORF in this frame can be exported directly into WinPep ("Export into WinPep" command in the "File" menu).

3.3. WinPep

3.3.1. Physicochemical Properties

A basic set of calculations determines some physicochemical properties of the given peptide, including amino acid composition, molecular weight, molar absorption coefficients for native *(3)* and denatured states *(8)*, and isoelectric point (cf. **Note 5**). Furthermore, the sequence can be searched for the occurrence of sequence motifs.

In the age of genomics, there is an increasing need for processing large groups of sequences instead of just individual ones. WinPep serves such demands by being able to process a file containing a basically unlimited number of polypeptide sequences in FASTA format and calculating the predicted physicochemical properties for all encoded polypeptides. Results are saved in a tab-delimited text file that can easily be read by any spreadsheet software such as MS Excel. Furthermore, some statistical parameters can be included in the output. The "Batch options" dialog allows specifying the features to be analyzed. The only field that is always present in the output file is the sequence name. By default, the settings of the user's last analysis are activated when the dialog box next time. It is possible to tabulate length, molecular weight, molar absorption coefficients, relative and absolute amino acid composition, isoelectric point, grand average of hydropathy (GRAVY, *(5)*), and number of predicted transmembrane helices. The following statistical parameters can be calculated for each molecular property: Mean, median, maximum, minimum, standard deviation and standard error.

If desired, a log file of the analysis is generated. This file includes date, name of the input file, name of the output file, number of successfully recognized sequences, used hydropathy scale for GRAVY, and used threshold value for identifying potential transmembrane helices. By default, analysis of a file "name.txt" generates the output file "name_results.txt" and "name_results_log.txt".

3.3.2. Sequence-specific Cleavage and the Identification of Posttranslational Modifications

For the simulation of sequence-specific cleavage, proteases and cleaving agents can be selected out of a provide list or can be defined individually. Use the "Select" or "Unselect" buttons to obtain a subgroup of agents to be used (cf. **Note 6**). The "Specificity" filed displays the specificity of the selected agents; user entry is not possible. Subsequently, either all possible fragments or only those from a complete digest are displayed (cf. **Note 7**).

The identification of potential posttranslational modifications is a function of WinPep2.11 and later. If mass spectroscopy yields peptides with an unexpected molecular weight, an algorithm tests whether this value might result from a posttranslational modification. First a simulation of a sequence-specific cleavage most be performed. The generated list of fragments is the basis for the subsequent analysis. WinPep displays a list of potential modifications. Furthermore, the presence of residues that could carry these modifications is indicated.

3.3.3. Helical Wheel

Part of the sequence can be displayed in a circular way with an offset of 100 degree, representing the typical α-helix. The view menu can be used to change the size of the image and to switch between color and monochrome display. In color mode, positively charged amino acids are red, negatively charged blue, hydrophilic uncharged green and hydrophobic uncharged gray (cf. **Note 8**).

Clicking with the right mouse-button invokes a floating menu where the displayed part of the sequence can be modified by opening the helical wheel dialog box. In addition, the amphipathy moment can be calculated. This quantitative value was defined to facilitate the identification of potential amphipathic helices: Considering the hydropathy of all amino acids within the given sequence window but not their distribution along the axis, the gradient across the axis is calculated. Accordingly, an amphypathy moment close to zero results from a relatively even distribution of hydrophilic and hydrophobic side chains while large values indicate clustering of hydrophilic and hydrophobic side chains on opposite sides of the helix. The amphypathy moment can be plotted along the entire sequence to further facilitate the identification of amphyphilic stretches. Thus, the amphypathy moment provides indications for further experimental validation.

3.3.4. Hydropathy Plots

Selected subsequences can be displayed as a hydropathy plot (cf. **Note 9**). For most scales, sequence stretches that average below zero are mainly hydrophilic and therefore likely to be surface-exposed, while stretches that average above zero are mainly hydrophobic and therefore likely to be buried or membrane-spanning.

Mean hydropathy (GRAVY score) and scale in use are indicated. Kyte and Doolittle *(5)* showed for their scale that none of 84 analyzed soluble proteins had a GRAVY score >0.5. Nevertheless, although a GRAVY-score >0.5 is a good indication that a protein is membrane bound, several membrane-embedded proteins have GRAVY scores below zero. Therefore, the amino acid sequence alone does not suffice to distinguish soluble from membrane-bound proteins *(5)*. If the scale by Kyte and Doolittle is used, putative transmembrane stretches are marked by a red line; the used threshold value is displayed next to the size of the sliding window. Kyte and Doolittle showed in a statistical analysis of several proteins that the mean hydropathy for the most hydrophobic 19-aminacid-stretch averages at 1.08 ± 0.22 for soluble and 1.86 ± 0.38 for membrane-bound proteins *(5)*. Therefore, they concluded that if a given 19-residue stretch has a mean hydropathy of at least 1.6 there is a high probability for membrane spanning. By default, WinPep uses a threshold of 1.6 to predict putative transmembrane segments. Nevertheless, it was also shown that the occurrence of multiple membrane-spanning segments often correlates with a lower hydrophobicity of the individual segments *(5)*. Therefore, the user might to modify the default hydropathy threshold.

Furthermore, individual residues can be marked by an arrow. Line color and font may be changed for each mark by double-clicking on it with the “Select” tool. Using the “Insert text” tool, user defined text items can be inserted. The position, font and content may be edited by clicking on them with the “Select” tool. Some text items are inserted by default (e.g. the currently used scale and mean hydropathy). They can be modified, repositioned or deleted by the user. However, once edited the automatic update of these items ceases.

Clicking the right mouse button invokes a floating menu where the displayed part of the sequence may be modified by opening the “Hydropathy plot properties” dialog. If the user has previously zoomed in, the display can be reset to the default view. In addition, results of selecting with the left mouse button may be toggled between zoom-in and opening a helical wheel display.

3.3.5. Domain drawing

A sketch of the domain composition of the sequence is easily generated using WinPep. Every display contains at least one domain – the entire sequence. Further domains can be added freely by the user. Domain color, name, font and text-color need to be specified. If domains overlap, domain defined last (i.e. last in the list) will appear on top of the others. Therefore, the shortest domain should be defined last. By changing the default scale, drawings of proteins of different lengths may be generated at a common scale. These individual drawings can be aligned in any graphic processing software. Individual residues can be marked. Line color and font are adjustable by double clicking on the mark. Furthermore, text items can be inserted and modified easily.

4. Notes

1. When sequences are pasted into WinGene or WinPep, any numbers, spaces or special characters will be deleted. Therefore, it is not require to remove sequence numbering manually beforehand.
2. Make sure to use the file in "gcg" format.
3. Installation of an updated "gcg.*" file may corrupt the existing YFE file by changing the numbering.
4. The minimal ORF length can be changed in the options menu.
5. The defaults pK_a values on which the calculations are based were taken from ***(6)***. However, these values can easily be changed in the options menu.
6. Available proteases can be edited and new ones added in the options menu.
7. Using the view menu, the list of fragments can be sorted by weight, start position or length.
8. The default settings for color coding amino acid residues can be changed for each helical wheel with the "Change colors" option in the options menu.
9. The program comes with two predefined scales ***(5,7)***. However, additional scales can be defined in the options menu.

Acknowledgments

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HPLC and Mass Spectrometry of Integral Membrane Proteins

Julian P. Whitelegge

1. Introduction

HPLC separations of membrane proteins can be conveniently divided into two categories. Firstly, there are many methods available for isolation of functional membrane proteins. Typically the proteins are maintained in configurations as close to their native state as possible through the use of mild detergents that provide solubility without denaturation, allowing convenient ion-exchange or size-exclusion chromatography, for example. Proteins or complexes isolated in this way are subsequently used for functional analysis or crystallization, and so on. These isolation techniques have been well reviewed and readers are referred to literature specific to the protein or membrane of interest. The second category of separations are those used to separate membrane proteins from detergents and salts for the purpose of protein chemistry; while tempting to call these methods "denaturing" there is substantial evidence that this is not always the case. The focus of this chapter is to review the latter category of HPLC techniques with specific reference to those methods that provide conditions compatible with mass spectrometric analysis, especially on-line electrospray ionization.

1.1. Biological Mass Spectrometry

The discovery of mild ionization techniques for mass spectrometry (MS) of biological macromolecules has revolutionized HPLC, since it is now convenient to monitor elution profiles not only by UV absorbance but by MS as well. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF ***(1)*** uses samples co-crystallized with a small UV absorbing matrix molecule to transfer molecules from a dried solid phase to the gas phase, and is thus not directly compatible with HPLC though spotting small aliquots (> 0.5 µL) of sequential fractions for

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MALDI-TOF remains a useful option. However, it was the application of electrospray-ionization (ESI) ***(2)*** that introduced the option of coupling HPLC directly to MS and the first liquid chromatography mass spectrometry (LC-MS) experiments provided an exciting breakthrough in technology ***(3).*** The HPLC eluent is directed straight to the ESI source for continuous ionization while the mass spectrometer measures mass to charge ratio (m/z) of ions detected during the course of a scan through the m/z range; a process that typically takes from 1 to 10 s. The read-out of mass spectra with time is thus complementary to the UV profile providing the opportunity to rapidly distinguish between different proteins and other UV absorbing molecules. As a consequence of the very low tolerance of MS to salts, LC-MS has been most widely applied using reverse-phase HPLC with volatile solvents (eg: acetonitrile) and ion-pairing agents (eg: TFA) though success is also achievable with size-exclusion HPLC provided salts are excluded. Variants of these methods have been successfully applied to a wide range of integral membrane proteins with structures containing up to fifteen transmembrane helices ***(4–7)***.

1.2. Mass Spectrometry of Membrane Proteins

Both ESI and MALDI-TOF have been applied to membrane proteins and peptides derived from them ***(4–13)***. The focus of this discussion will be ESI in the context of on-line LC-MS since it provides superior mass accuracy and resolution compared to MALDI-TOF, especially for intact proteins. The ability to use ESI-MS for analysis of integral membrane proteins was first demonstrated in 1993 ***(9, 10)*** using flow-injection methods. LC-MS was applied in 1997 ***(14)*** though the gradient elution with undiluted formic acid can be regarded as somewhat extreme and mass accuracies in the 0.01–0.05% range were lower than obtained for water-soluble proteins. The adoption of a reverse-phase system involving elevated concentrations of aqueous formic acid (60%) and gradient elution with isopropanol allowed routine achievement of 0.01% accuracy ***(4)***. A size-exclusion LC-MS method for intrinsic membrane proteins was reported in 1999 ***(5)*** and has proved broadly applicable ***(6, 7)*** again with routine achievement of 0.01% accuracy (+/− 9 Da at 90 kDa). Figure 1 shows the results of a size-exclusion LC-MS experiment performed upon bacteriorhodopsin. The mass spectrum **(Fig. 1A)** collected during elution of the protein shows a characteristic

→

Fig. 1. (continued) (protons) associated with a particular protein molecule, in this case from 15 to 29 with the most abundant at M + 24 H^+. The singly charged CHAPS molecules presumably remained associated with the protein during chromatography but were separated during ionization. Minor signals at the center of the mass spectrum result from minor isoforms of the protein that are more clearly visualized in the molecular weight spectrum (B). Note resolution of lesser sub-populations of all species that measure 16–18 Da larger and probably correspond to singly oxidized species (+ O, 16 Da) or possibly carry a single bound water (+ 18 Da). Running the column at the usual operating temperature of 40 °C results in predominance of the apoprotein.

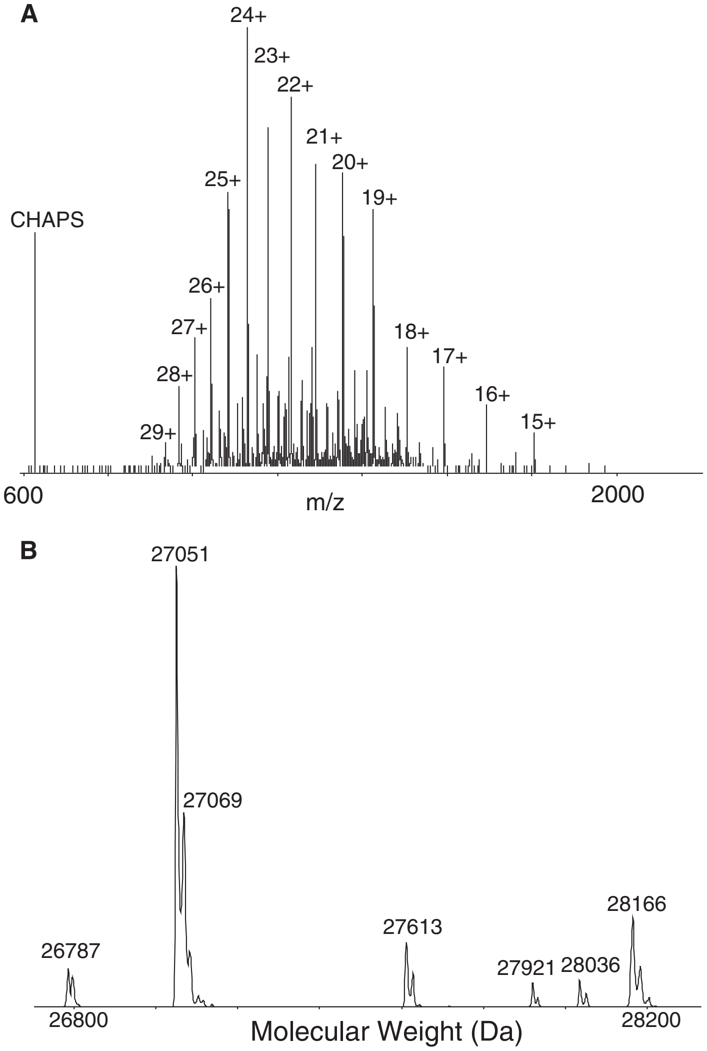

Fig. 1. Electrospray-ionization mass spectrometry of bacteriorhodopsin via online size-exclusion HPLC. Bacteriorhodopsin (Sigma, 50 µg) suspended in 1 mM CHAPS was acidified by addition of two volumes of formic acid/isopropanol (1/1, v/v) and loaded immediately onto an HPLC system with a size-exclusion column (Super SW 2000, 4.6 × 300 mm; Tosohaas) equilibrated in chloroform/methanol/1% aqueous formic acid (4/4/1, v/v; 250 µL/min.; 24°C). Eluent was passed through a UV detector and directly to an electrospray-ionization source (Ionspray™) of a triple quadrupole mass spectrometer (API III, PE Sciex). The instrument was operated with an orifice potential of 65 V and scanned from m/z 600–2300 with a step size of 0.3 and a dwell of 1 ms to give a scan time of 6 s. Scans collected during the elution of the first major protein peak were averaged **(A)** and processed by computer to generate a zero-charge spectrum **(B)**. Multiple charging during the electrospray process generates a cluster of ions that differ in m/z as a consequence of the number of charges

cluster of multiply-charged ions corresponding to the protein itself (15–29 H^+) and the singly-charged ion derived from the detergent CHAPS. A computerized reconstruction of the zero-charge molecular weight spectrum **(Fig. 1B)** shows predominance of the holoprotein (27051 Da; calculated 27050.06 Da), a small amount of apoprotein (26787 Da; calculated 26783.64 Da) and other minor species that probably correspond to incompletely processed forms of bacteriorhodopsin that have nevertheless bound the retinal cofactor.

1.3. Proteomics

While many proteomics studies are using 2-D gel electrophoresis as the primary separation technology there are recognized disadvantages of applying these methods to some classes of integral membrane proteins ***(15)***. Therefore, alternative technologies are sought with HPLC separations playing a potentially important role. **Fig. 2** shows a modification of LC-MS for use in proteomics. By inserting a liquid-flow splitter between HPLC and mass spectrometer it is

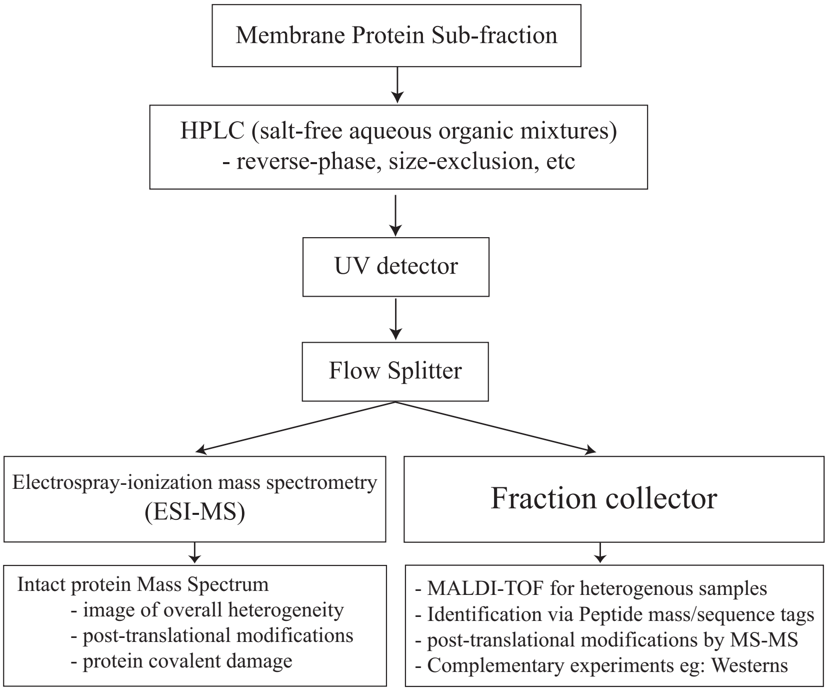
Membrane Protein Sub-fraction
HPLC (salt-free aqueous organic mixtures)
- reverse-phase, size-exclusion, etc
UV detector
Flow Splitter
Electrospray-ionization mass spectrometry
(ESI-MS)
Fraction collector
Intact protein Mass Spectrum
- image of overall heterogeneity
- post-translational modifications
- protein covalent damage
- MALDI-TOF for heterogenous samples
- Identification via Peptide mass/sequence tags
- post-translational modifications by MS-MS
- Complementary experiments eg: Westerns

Fig. 2. Liquid chromatography with mass spectrometry and fraction collection (LC-MS+). Use of a flow splitter after the HPLC detector allows concomitant mass spectrometry and fraction collection. Fractions can be analyzed after analysis of intact protein mass spectra to allow increased confidence of identification and characterization of post-translational modifications.

possible to collect fractions concomitant with MS (LC-MS+) which can be used in downstream experiments for protein identification and characterization of post-translational modifications. This approach was applied successfully to the cytochrome b_6f complex from higher plants, identifying a ninth subunit as ferredoxin/NADP$^+$ oxidoreductase (FNR) ***(16).*** Since typical membrane preparations are too complex to fully resolve by a single chromatographic separation, it is suggested that sub-fractions should be employed ***(17).***

1.4. Solubility and Aggregation of Membrane Proteins

Following extraction from bilayers, membrane proteins have an extreme tendency to aggregate and precipitate unless stabilized in detergent micelles. For HPLC separations in the absence of detergent it is then necessary to find solvent conditions compatible with both solubility and dis-aggregation of the analyte. During attempts to isolate hydrophobic peptides derived from proteolytic cleavage of the photoaffinity-labeled D1 herbicide receptor ***(18)*** it was observed that the labeled peptide of interest would only be *retained* in size-exclusion HPLC using 50% formic acid, 50% methanol as mobile phase. The observation that the peptide eluted in the void when 50% aqueous formic acid or 50% aqueous acetic acid or 50% acetic acid, 50% methanol were used as mobile phase emphasized that both solubility *and* dis-aggregation were necessary for chromatographic separation. While formic acid is a very useful reagent for solubilization/dis-aggregation it should be recognized that at high concentrations it will modify proteins with formyl groups that appear as multiple + 28 Da adducts in the mass spectrum. Consequently, more recent protocols, while using high concentrations of formic acid for initial solubilization, emphasize immediate transfer to more inert solvents.

2. Materials

2.1. Hardware

1. HPLC pumps/plumbing:

The techniques described here use standard HPLC systems, with syringe pump instruments for flow rates less than 200 µL/min. High concentrations of formic acid (60–90%) are rinsed promptly from pumps/syringes but are probably no more corrosive than 1 M NaCl, for example. High-pressure lines use stainless steel tubing and ferrules but lower pressure lines use Peak™ tubing and Fingertight™ fittings (< 2000 psi). Fused-silica capillaries (PolyMicro) are used to interface LC to the ESI source using minimal length connections due to the possibility of chromatography on the inner glass surface. All systems are modified to minimize liquid paths and dead volumes of connections. Absorbance is measured at 214 nm or 280 nm if solvent background is prohibitive at 214 nm (high [formic acid]; chloroform, and so on). Note that some flow cells are sensitive to excessive back-pressure and that it is sometimes necessary to use splitters or wider bore tubing to avoid expensive accidents. HPLC systems typically need servicing after 2–3 years of moderately heavy use but need no specialized treatment for the methods described. An oven is often used in order to run columns at elevated temperatures.

2. Syringe pumps

HPLC systems with syringe pumps are typically used for LC-MS at lower flow rates. Well-serviced machines provide good performance provided care is taken to ensure buffers/solvents are thoroughly degassed. Pistons and seals are replaced when necessary. Syringes are typically purged empty at the end of the day. Sometimes specific problems can be overcome by appropriate modification. For example, an ABI 120A being used for LC-MS work had a static mixer (a small C18 column) placed before the purge valve that resulted in an over-pressure condition when purging high viscosity solvents such as 60% formic acid/isopropanol. The static mixer column was consequently situated after the purge valve such that it was eliminated from the purge cycle.

3. Columns

For reverse-phase chromatography, polystyrene-divinylbenzene copolymer is preferred over silica-based columns. This media can be regenerated more thoroughly and run at higher temperatures. PLRP/S (Varian Inc.) provides excellent chromatographic performance while PRP (Hamilton) provides an acceptable less-expensive alternative. For size-exclusion chromatography, the SW series of silica supports (Tosoh Biosciences) provides the only suitable media. Several other companies have advertised equivalent performance but tests have shown that only the Tosoh product performs adequately in chloroform/methanol/1% aqueous formic acid (4/4/1). While this solvent exceeds the manufacturers recommended organic percentage limit we experience excellent longevity and the column can be stored in this solvent. Elevated concentrations of formic acid shorten PLRP/S column life significantly. Virgin columns provide ultimate chromatographic resolution and researchers should not expect the extended lifetime achievable when these columns are used for peptides. A cost of around $50 per membrane protein experiment is projected. Guard columns are not used but the column is protected with a post-injector 0.2 μm filter.

4. Grounding and safety

Use of electrospray ionization involves high potential differences between source and mass spectrometer that can be conducted down solvent lines. Thus the HPLC stack must be grounded to the mass spectrometer. Furthermore, source voltage must be removed prior to manipulation of solvent lines to protect the operator. Protective clothing and eyewear must be used when working with pressurized solvents and acids. An extractor at the ESI source is also useful to avoid accumulation of solvent/acid vapors.

2.2. Chemicals

The use of MS with HPLC makes the operator very aware of the quality of solvents and chemicals being used. Remarkable differences in chemical background can be observed with different suppliers. Thus different batches or alternative supplies are tested carefully in the chromatographic systems of interest to ensure low background.

1. Trifluoroacetic acid (TFA) is from Pierce (1 mL sealed ampules of Ionate™ grade).
2. Formic acid is from Fisher (90%, ACS grade) and is not stored for long periods (*see* **Note 1**).
3. Water is double distilled in Quartz or from a Millipore reverse osmosis system (> 18 MegaOhm).
4. Organic solvents are from Fisher; methanol is HPLC grade, acetonitrile is Optima grade and chloroform/isopropanol are ACS grade. Solvents are mixed in glass (try LC-MS with a chloroform containing solvent that was mixed in plastic for your amusement) and degassed briefly by sonication under vacuum. Solvent mixtures containing chloroform or isopropanol are made up daily whereas standard TFA buffers may be kept for at least one week. Aqueous TFA supports microbial life and is usually discarded after two weeks. Note that halogenated solvent waste is generated by nearly all the methods described and should be disposed of properly.

3. Methods

3.1. Sample Purification

There are numerous methods for isolation of membrane fractions, membrane protein complexes and monomeric membrane proteins but these are not the focus of this chapter and are covered by many other authors.

3.2. Sample Preparation

Once a membrane protein fraction has been isolated in a functional state, it is typically accompanied either by native lipids or detergents. Sometimes it may be possible to load a sample in this state and use the chromatography to separate the protein from these contaminants. Often however, small amphiphiles remain associated with and/or coelute with the protein and interfere with chromatography/mass spectrometry. Thus it is usual to precipitate the protein with organic solvents in order to remove contaminating lipids/detergents, or at least deplete them, prior to chromatography. In the case of the chlorophyll-binding proteins of the thylakoid membrane the pigment must be extracted for successful separations, easily achievable by acetone precipitation.

3.2.1. Precipitation with acetone.

Acetone precipitation is achieved at 80% concentration by volume at −20°C. Either add acetone to 80% or add 80% acetone to a small sample volume. Stocks should be at −20°C. Precipitation usually occurs rapidly within 10 min at −20°C but can take longer. Concentrated samples may work better if aliquoted out whereas dilute samples may be lost. Some lipids will remain associated with precipitated protein. Detergents interfere with acetone precipitation dramatically and should be avoided. If detergents are present, protein should be precipitated at the interface of a chloroform/methanol/water phase separation. Precipitates are recovered by brief centrifugation (10,000 × g; 1 min.).

3.2.2. Precipitation with chloroform/methanol/water phase separation

The protocol, modified from Wessel and Flugge ***(19),*** for this precipitation is shown in **Fig. 3** and represents an important breakthrough in membrane protein chemistry. Precipitation at the interface is usually efficient even in the presence of 2% SDS/ 1% Triton X100™ in the sample. Of course overall yields vary with sample type and amount of detergent. More lipid is removed than with acetone precipitation though some may remain. The most hydrophobic of proteins can partition into the lower phase; these are the proteolipids and can be recovered by size-exclusion chromatography **(Subheading 3.3)** or by precipitation with ether at low temperature.

3.2.3. No Precipitation

Precipitation can be disadvantageous and even lead to covalent modification of the sample. A good example is bacteriorhodopsin, a seven-transmembrane protein that carries a Schiff base linked retinal cofactor that is central to its function as a light-driven proton pump ***(20).*** Initial success in analysis of the protein by LC-MS was achieved after precipitation of purple membrane with acetone, solubilization in formic acid/water/isopropanol and LC-MS in the 60% formic acid/isopropanol system **(Subheading 3.2)** though the majority of the protein was recovered as the apoprotein. When the sample, instead of acetone precipitation, was dispersed in detergent (1 mM CHAPS) and acidified with formic acid/isopropanol (three volumes of 1:1) prior to LC-MS, nearly complete recovery of the holoprotein resulted. It is believed that stabilization of the Schiff-base results due to retention of some secondary/tertiary structure ***(4),*** despite what seem rather harsh conditions. Addition of four volumes of 90% formic acid is routinely used in today's protocol for "purple membrane." Some bacteriorhodopsin may be captured on the filter used to protect the columns and can be removed with follow up formic acid injections.

3.2.4. Dissolution

Following precipitation with organic solvents, samples are typically dried for about two minutes at room temperature and atmospheric pressure. This is to allow evaporation of excess organic solvents but not water. Thorough drying at this

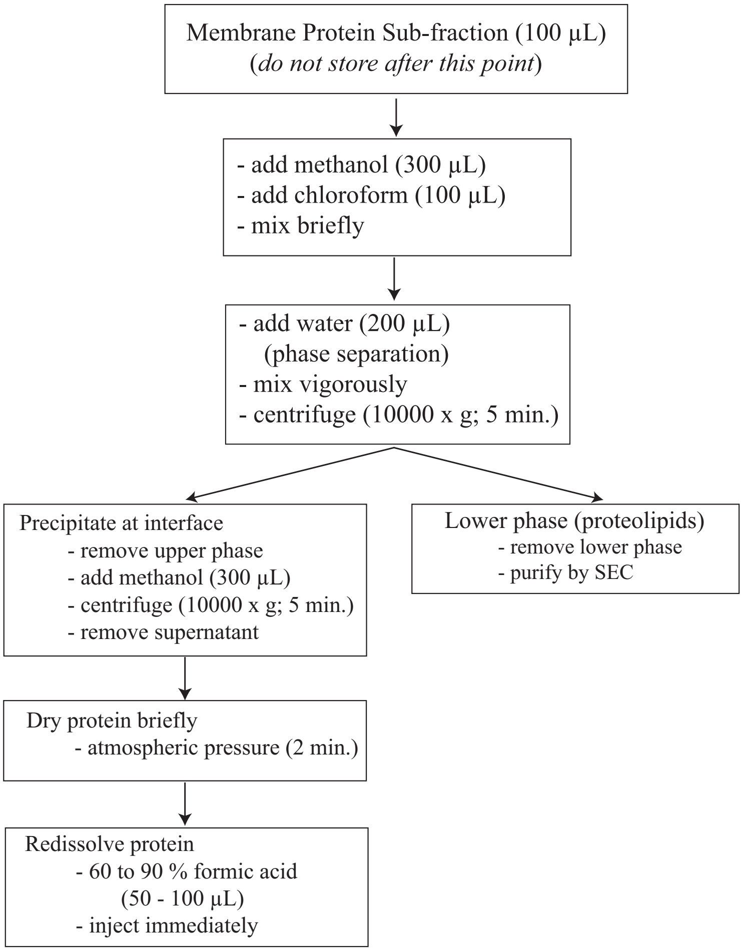
Membrane Protein Sub-fraction (100 μL)
(do not store after this point)
- add methanol (300 μL)
- add chloroform (100 μL)
- mix briefly
- add water (200 μL)
(phase separation)
- mix vigorously
- centrifuge (10000 x g; 5 min.)
Precipitate at interface
- remove upper phase
- add methanol (300 μL)
- centrifuge (10000 x g; 5 min.)
- remove supernatant
Lower phase (proteolipids)
- remove lower phase
- purify by SEC
Dry protein briefly
- atmospheric pressure (2 min.)
Redissolve protein
- 60 to 90 % formic acid
(50 - 100 μL)
- inject immediately

Fig. 3. Chloroform/methanol/water phase separation for precipitation of protein and removal of lipids and detergents. Protein can be precipitated from remarkably high concentrations of detergents. Procedure modified from Wessel and Flugge ***(19)***.

stage results in pellets that are more resistant to redissolution. A small aliquot of solvent (50–100 μL) is added to the "wet" pellet for immediate redissolution and injection to HPLC. The solvent used most frequently is 60% formic acid but 90% formic acid was necessary to dissolve the *E. coli* lactose permease. Acetic acid can often be substituted for formic acid. TFA (50–100%) is also effective for solubilization though there is increased tendency for backbone hydrolysis in this strong acid. Solubility is sometimes influenced by additions of organic solvents, such as isopropanol, trifluoroethanol or hexafluoroisopropanol at this stage though it should be noted that such additions may lead to *decreased* as well as increased solubility. Conditions for specific samples are best determined empirically. Furthermore solubility is influenced by the precipitation protocol used, which influences degrees of denaturation and delipidation, as well as specifics such as the actual concentration of protein during precipitation. It is suggested that extreme attention to detail is applied to all aspects of the sample preparation protocol with any storage steps minimized especially after removal of lipids/detergents (*see also* **Note 3**).

In cases where samples were not precipitated it is usual to acidify the sample prior to injection, with four volumes of formic acid, for example. If the sample is not acidified it will, in specific cases, remain bound to the column until an aliquot of the appropriate acid is passed down the column. This behavior can be used to advantage for on-column delipidation or detergent removal, for example.

3.3. Reverse-Phase HPLC (RP-HPLC).

The compatibility of the solvent systems used in reverse-phase with LC-MS combined with the often superior chromatographic resolution makes this a frequently used option for HPLC of integral membrane proteins, especially when a mixture is present. Many have described standard reverse-phase chromatographic systems and the first system to be described here is the author's version that includes a polymeric stationary phase and has given satisfactory results for many integral membrane proteins.

3.3.1. TFA/Acetonitrile System

While many intrinsic membrane proteins elute efficiently from a reverse-phase stationary phase with the TFA/acetonitrile system, there are some proteins that have very low elution efficiencies such that little or no protein is observed to elute at all. These are typically the most hydrophobic, such as bacteriorhodopsin. However, it should be appreciated that this behavior provides the opportunity to wash proteins of lipids and detergents on-column and to specifically enrich these molecules on-column, for subsequent elution with secondary gradients of more powerful eluents.

1. Buffer A is 0.1% TFA in water, buffer B is 0.1% TFA in acetonitrile. Buffers are degassed prior to use, typically by sonication and vacuum aspiration (with trap).
2. Column (PLRP/S, Varian Inc.; 300 Å, 5 µm; 2 × 150 mm; 40°C) is equilibrated in 5% B for 10 minutes (100 µL/min).
3. Sample injection (100 µL loop) initiates gradient program. 5% B for 5 minutes followed by a linear gradient from 5–100% B over 60 minutes. System runs at 100% B for 5 minutes and returns to 50% B (syringe pumps) or 5% B (regular pumps) over 5 minutes. Gradient may be adjusted for optimal separations of specific samples.
4. A preliminary run with no injection should show a flat baseline. A second preliminary run with injection of formic acid (100 µL) will then reveal potential ghosts

from previously retained proteins as well as UV absorbing material contaminating the formic acid. Mass spectrometry (ESI-MS) can be used to distinguish ghosts from UV absorbing material of nonprotein origin. If ghosts are detected the column must be regenerated/replaced.

5. Dual wavelength detection at 214 and 280 nm.
6. Column regeneration (*see* **Note 2**).

3.3.2. TFA/Acetonitrile/Isopropanol System

The addition of isopropanol to acetonitrile has been known to improve elution efficiency of many integral membrane proteins in reverse-phase chromatography for many years ***(21)*,** enabling the purification of rhodopsin, for example. Bacteriorhodopsin elutes quite efficiently allowing fully resolved spectra of many different isoforms of widely varied abundance.

1. As above but buffer B is 0.05% TFA in acetonitrile/isopropanol (1:1 by volume). Buffer B is prepared freshly daily in glass by mixing equal volumes of 0.1% TFA in acetonitrile and isopropanol. Use glass stoppers on HPLC bottles rather than plastic lids that will leach plasticizers.
2. Gradients may be adjusted to focus upon the 50–100% B part of the chromatogram where the most hydrophobic of the integral membrane proteins elute (*see* ***Ref. (23)***).
3. Column regeneration (*see* **Note 2**).

3.3.3. Formic Acid/Isopropanol System

While addition of isopropanol to acetonitrile often improves elution efficiency, there will most usually be some residual intrinsic membrane protein remaining column bound. A solvent system with elevated formic acid concentration in the polar phase and isopropanol as the eluting solvent was originally described for separation of poliovirus proteins with quantitative recovery of material ***(22)*.** Most intrinsic membrane proteins elute with close to 100% efficiency in the 60% formic acid/isopropanol system described here though exceptions are possible. The major subunit of *Torpedo* Vo ATPase (four transmembrane helices) has reduced elution efficiency and the lactose permease (twelve transmembrane helices) from *E.coli* was not recovered at all under these conditions. In both examples elevation of formic acid concentration to 90% allowed full recovery of the proteins. The major disadvantage of the technique is the potential for protein formylation that results in multiple +28 Da adducts, very apparent in protein ESI mass spectra. Protein exposure to formic acid should be minimized or avoided. Acetic acid may provide a suitable alternative in some cases.

1. Buffer A is 60% formic acid, buffer B is isopropanol. Buffers are degassed prior to use, typically by sonication and vacuum aspiration (with trap).
2. Column (PLRP/S, Polymer Lab.s; 300 Å, 5 μm; 2 × 150 mm; 40°C) is equilibrated in 5% B for 10 minutes (100 μL/min).

3. Sample injection (100 µL loop) initiates gradient program. 5% B for 5 minutes followed by a linear gradient from 5–100% B over 40 minutes. System runs at 100% B for 5 minutes and returns to 50% B (syringe pumps) or 5% B (regular pumps) over 5 minutes. Extended runs should be avoided due to the potential for sample formylation.
4. Detection is at 280 nm.
5. Column regeneration (*see* **Note 2**).

Size-Exclusion Cromatography (HPLC-SEC)

While the 47 kDa *E.coli* lactose permease could be eluted in reverse-phase by increasing the formic acid concentration to 90%, the resulting mass spectrum showed multiple formylation adducts resulting in over estimate of mass by 100–150 Da. Thus an alternative chromatographic separation was sought that would permit separation of the purified permease from residual detergent/lipid and the multitude of small molecules contaminating 90% formic acid. John E. Walker had previously reported success with ESI of mitochondrial inner membrane proteins using a solvent system containing chloroform/methanol/1% aqueous formic acid (4/4/1, v/v) ***(13)*** though separations were performed off-line prior to ESI-MS. Based upon previous success in size-exclusion HPLC with Tosoh silica SW media using formic acid/methanol (1/1, v/v) ***(18)***, the Walker solvent system was substituted. The precipitated permease was dissolved in 90% formic acid and immediately injected onto a SEC LC-MS system using an SW2000 column equilibrated in the 4/4/1 solvent mixture. Rather than precipitating as expected, the protein was quickly transferred to the running solvent. Small molecule contaminants were retained and the permease eluted before them, close to the void, allowing spectra to be recorded with excellent signal/noise ratio ***(5)***. The SEC-MS system described has proved robust allowing general success with a variety of membrane and other proteins ***(6, 7)***. The main limitation of the technique is the lack of chromatographic resolution afforded by SEC such that samples must be quite highly purified. The thylakoid cytochrome $b_6 f$ complex provided a good example of the complexity of the mixture that could be resolved. Five larger subunits coeluted but could be recognized in the rather complex mass spectrum since small subunits and small molecule contaminants were more highly retained and thus eliminated from the spectrum ***(16, 23)***. A typical elution profile is shown in **Fig. 4**. Protein elutes first followed by smaller UV absorbing molecules **(Fig. 4A)**. The total ion chromatogram is a read-out of total mass spectrometer signal with time and shows the separation of the protein (concomitant with A_{280}) from other small molecules that give strong signals during mass spectrometry and would spoil the protein mass spectrum unless removed.

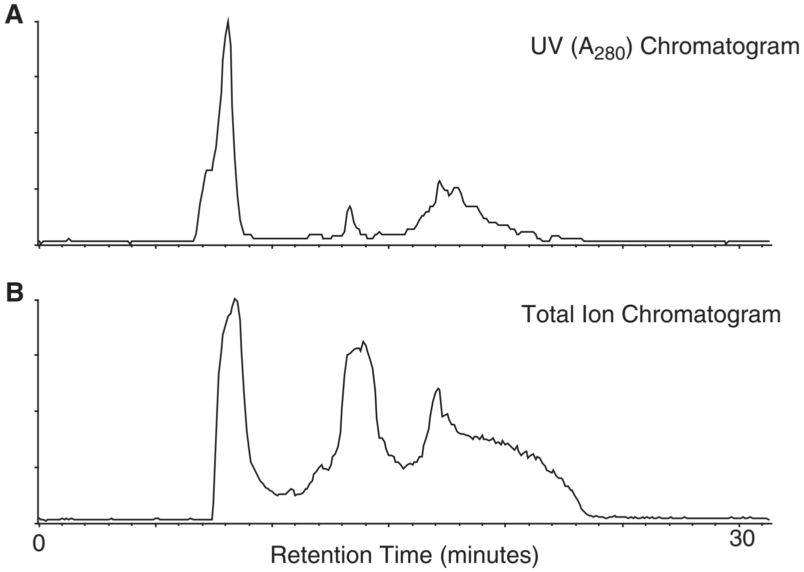

Fig. 4. Elution profile during size-exclusion liquid chromatography mass spectrometry (LC-MS). A protein sample (100 µg KcsA in 25 µL; a kind gift of Chris Miller, Brandeis University) was acidified (75 µL 90% formic acid) and subjected to size-exclusion chromatography mass spectrometry on Super SW2000 (Tosohaas; 4.6 × 300 mm) equilibrated in chloroform/methanol/1% aqueous formic acid (4/4/1; v/v) at a flow rate of 250 µL/min and 40°C. The absorbance profile at 280 nm **(A)** is compared to the total ion chromatogram **(B)**. The total ion chromatogram is generated by summing the total signal in one scan of the mass spectrometer (6 s) and plotting against time. Note the appearance of strong mass spectrometric response concomitant with the first UV peak as the protein elutes. The forward shoulder on the UV profile is probably a result of some protein running in an aggregated or oligomeric state and thus eluting in the void. The protein is clearly separated from other molecules that absorb UV light and exhibit strong response in mass spectrometry, with retention times greater than 10 min.

1. Buffer is chloroform/methanol/1% formic acid in water (4:4:1; by volume) and degassed as above. The solvent is prepared freshly each day and mixed/stored in glass containers.
2. Column (Tosoh Biosciences, Super SW2000, 4.6 × 300 mm; 40°C) is equilibrated for several column volumes (250 µL/min) until ESI-MS reveals drop in chemical background to very low levels. Note that the columns ship in sulfate/phosphate containing buffers which must be thoroughly washed away with water prior to transfer of the column first to 80% methanol and then the final solvent.
3. Sample is introduced with a 100 µL loop initiating the run which is complete within 35 min unless large amounts of small molecules such as detergents have been

introduced. Complete removal of excessive SDS or salt contamination can take days of washing.

4. Columns may be connected in series for improved chromatographic resolution.
5. Detection is at 280 nm.
6. Column regeneration (*see* **Note 2**).

3.4. UV Detection with Mass Spectrometry

The availability of mass spectrometers able to tolerate a continuous flow of liquid to the source enabled the advent of liquid chromatography mass spectrometry (LC-MS) *(3)*. Not only has this development revolutionized biological mass spectrometry but also HPLC. The eluent from the HPLC UV detector is directed to the electrospray ionization source of the mass spectrometer that thus becomes an on-line detector **(Fig. 1)**. The combination of UV and mass spectral data provides vastly improved detection because proteins are not only visualized by their general UV profile but also their unique mass spectral profile including covalent heterogeneity. Many protein modifications can change retention properties and thus mass spectral information becomes essential for interpreting data. An excellent illustration of this point is provided by a study that used changes in retention time to conclude that formic acid treatment leads to reduction of disulfide bonds within insulin and other proteins. When insulin was treated with formic acid there were changes in retention time to values similar to the individual α and β subunits. However, repetition of these experiments using LC-MS revealed formylation of the intact insulin molecule with corresponding changes of retention time (Tjon, Stevens, Faull and Whitelegge, unpublished data). Reliance upon retention time alone had led to complete misinterpretation of the data. Though listed as a strong reducing agent by the Merck index, we have found no evidence of disulfide reduction by formic acid in several different proteins.

Many are tempted to collect fractions and perform mass spectrometry later. While such an approach may be tolerated where necessary, many benefits of LC-MS are lost. Perhaps the most significant of these is the very low chemical noise that a well set up LC-MS system provides though loss of material due to adsorption, loss of solubility and covalent modification can all contribute to dilution of benefits.

3.5. Sample Recovery and Tertiary Structure

If it is necessary to collect fractions for further experiments, the benefits of LC-MS can be retained through the use of a splitter as described (LC-MS+; **Subheading 1.3**). If yield must be maximized then the whole sample can be collected at the expense of mass spectrometric detection. For structural measurements samples can be dried onto Germanium chips for attenuated total reflectance

Fourier transform infrared (ATR-FTIR) spectroscopy; both bacteriorhodopsin dried down from the 60% formic acid/isopropanol system (Whitelegge and Waring, unpublished) as well as the lactose permease dried down from the chloroform/methanol/1% aqueous formic acid system (Whitelegge, le Coutre and Kaback, unpublished) showed FTIR spectra indicative of substantial α-helix, supporting the possibility of retention of structure in these solvents.

3.6. The Future

While the techniques/protocols above provide a sound starting point for quite highly purified preparations there is a great need for techniques that provide superior resolution in separations of all intrinsic membrane proteins and especially crude mixtures. There are a wealth of different surface chemistries and solvent mixtures available for HPLC and LC-MS providing a huge practical area for further exploration. A recent paper from J.E. Walker's group highlights the considerable progress that has been made in the analysis of intact mitochondrial membrane proteins and includes a novel hydrophilic interaction chromatography (HILIC) protocol that will be interesting to explore in other membrane systems ***(24)***. There will be a dramatic expansion in top-down mass spectrometry (intact proteins are fragmented in the mass spectrometer) in the next few years and the techniques discussed herein are all compatible with this process – early results suggest great promise for the study of integral membrane proteins **(23–25)**.

4. Notes

1. Formic Acid.Considerable attention should be paid to formic acid stocks used for protein chemistry. The reagent should be clear and colorless as supplied. Aging in the laboratory leads to a yellowish/brown appearance than can become intense after prolonged storage. Unfortunately, formic acid is typically shipped in plastic bottles that leach plasticizers readily. UV 280 absorbing contaminants as well as molecules that ionize readily during ESI accumulate steadily with storage, contributing to chemical background. While chromatographic separations can be relied upon for separation of such contaminants from molecules of interest, it is preferable to replace stocks regularly and to test each new stock in case it sat in a warehouse for some months before delivery. Different sources of formic acid show varying potentials for protein formylation and the relationship between this activity and contaminants is not clear. Despite these drawbacks, formic acid is never-the-less most useful for membrane protein chemistry.
2. Column Regeneration. Reverse-phase columns were originally regenerated by running gradients of formic acid/isopropanol (A: 90% formic acid, as supplied by Fisher, undiluted; B: isopropanol) from 5% B to 100% B

with prolonged washing for 1 h at 100% B. While effective for regenerating the columns, this protocol shortens the life of polymeric columns substantially. Now reverse-phase columns are equilibrated first in 80% methanol and then chloroform/methanol/1% aqueous formic acid (4/4/1). Aliquots of undiluted formic acid are injected in a repetitive fashion until LC-MS shows that such injections give no further release of protein. For regeneration of the silica Tosohaas SEC columns, the same latter protocol is used. If protein contamination is particularly tenacious with substantial elution of protein even after say five formic acid injections, the column can be regenerated with 90% formic acid/[chloroform/methanol/water; 4/4/1] (8:1, v/v) for one column volume, though this wash is avoided accept where absolutely necessary.

3. Pressure-Assisted Solubilization. During analysis of samples of bovine rod outer-segment membranes, highly enriched in the G-protein coupled receptor rhodopsin, it was common to observe a dramatic increase in system pressure upon injection of sample. Typically 2000–2500 psi could be reached before a sudden return of the system to standard operating pressure occurred. While we have not investigated this phenomenon further, a reasonable explanation is that elevated pressure played a role in transferring the rhodopsin protein to the soluble/retained phase prior to successful elution in the gradient.

Acknowledgements

The techniques described here are evolved from huge amounts of groundwork by many others, especially Gobind Khorana and John Findlay. My career in HPLC and mass spectrometry would not have been possible without the generous mentorship of Kym Faull and my colleagues at UCLA. I have learnt hugely from my peers and I acknowledge invaluable contributions from Sir John E. Walker, Ian Fearnley, H. Ron Kaback, and Bill Cramer amongst many others to whom I apologize for not mentioning. I specifically thank John R. Bowyer and Patrick Camilleri who understood and shared my interest in techniques compatible with mass spectrometry and Chris Gerrish for his skillful help with HPLCs.

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Enrichment of Serum Peptides and Analysis by MALDI-TOF Mass Spectrometry

Yanming An, Habtom W. Ressom, and Radoslav Goldman

1. Introduction

Serum and plasma are readily available patient samples frequently utilized in measurement of disease markers ***(1)***. The presence of peptides in serum and plasma reflecting various disease processes was demonstrated in several recent studies ***(2–9)***. The combination of improved mass spectrometric and sample preparation methods allows gradual improvement in peptidomic marker discovery. The various solutions to marker discovery search for an ideal compromise in throughput required to compare a sufficient number of patient samples and in resolution allowing quantification of low abundant analytes. A comprehensive quantitative comparison of low abundant peptides expected to exist in connection with disease processes continues to present a significant challenge ***(10–11)***. In this chapter, we describe a method for concentration and desalting of serum peptides on magnetic beads that reduces signal suppression in subsequent MALDI-TOF mass spectrometric quantification of the low mass analytes. Further removal of high abundant serum proteins by denaturing ultrafiltration narrows the dynamic range of the complex mixture ***(12)***. The magnetic beads are coated with functional groups such as reverse phase C8, commonly used in liquid chromatographic columns. Recovery of peptides from magnetic beads can be automated to allow a reproducible processing of a relatively large number of patient samples. The resulting micro volumes of purified peptides are conveniently analyzed by MALDI-TOF/TOF or other proteomic strategies.

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2. Materials

2.1. Blood Sample Collection and Serum Preparation

1. Vacutainer serum tubes (BD 366430) (Becton Dickenson, Franklin Lakes, NJ)
2. Vacutainer Safety-Lok blood collection set (BD 367281)
3. Vacutainer holder (BD 364815)
4. NUNC cryotubes (Nalge Nunc International, Rochester, NY, Cat. No. 377267)

2.2. Capture of Peptides on Magnetic Beads

1. Purification Kit 1000 MB HIC 8 (Bruker Daltonics, Part No. 223324)

The kit includes Binding Solution, Washing Solution, magnetic beads C8, and water.

2. Trifluoroacetic acid (TFA) (Sigma-Aldrich) (Highly corrosive)
3. Acetonitrile CHROMASOLV Plus, for HPLC ≥99.9% (Sigma-Aldrich) (Highly flammable and harmful)
4. 0.2 mL low profile thin-walled 8-tube & flat cap strips (ABgene Cat. No. AB-0773)
5. Radio-Immuno-Assay tube (Sarstedt Aktiengesellschaft & Co. Germany, D-51588)
6. The Magtration SX-8G sample processing robot (Precision System Sciences USA, Inc., Livermore, CA) (*see* **Note 1**)
7. DN 70 pipette tip with filter (Precision System Sciences USA, Cat. No. F4403)
8. MagnetSeparator 104 II (Bruker Daltonics, Part No. 207151)
9. Serum samples (see blood collection)

2.3. Ultrafiltration

1. Microcon ultrafiltration units with MWCO 30 kDa (Millipore, MA)
2. Water CHROMASOLV Plus, for HPLC (Sigma-Aldrich)

2.4. MALDI Mass Spectrometry

1. α-Cyano-4-hydroxy-cinnamic acid (CHCA), 3.3 mg/ml (Bruker Daltonics) (*see* **Note 2**)
2. MTP AnchorChip ™600/384 T F (Bruker Daltonics, Part No. 209513)
3. Peptide calibration standard (Bruker Daltonics, Part No. 206195) (Store at −20°C)
4. Sample buffer: 5% acetonitrile with 0.1% TFA (47.5 ml water, 2.5 ml acetonitrile and 50 µL TFA)

3. Method

3.1. Blood Samples Collection and Serum Preparation

1. Blood samples collected in a BD Vacutainer "red-top" tube according to a standard manufacturer's protocol are allowed to clot for 60 min (*see* **Note 3**).
2. Serum is separated by centrifugation at 1,200 g for 10 min and aliquoted as soon as possible in a convenient volume for freezing at −80°C in NUNC Cryotubes (*see* **Note 4**).
3. At first thaw, serum is sub-aliquoted at 50 µL to store at −80°C until processing. All samples are processed at second thaw (*see* **Note 5**).
4. Thawing of serum aliquot is achieved by submersion in room temperature water bath; samples are processed as soon as possible after thawing.

5. Particulates from serum are removed by a spin in a tabletop centrifuge at ~10,000 g for 10–15 s.

3.2. Robotic Protocol

We describe a protocol using an SX-8G laboratory automation system dedicated to the handling of magnetic beads (Precision System Sciences USA, Inc., Livermore, CA). The SX-8G robot uses an 8-channel Magtration ® pipetting unit for separation of magnetic beads from sample volumes of 5 to 200 µL. We describe a program for a simultaneous handling of 8 samples (*see* **Note 6**).

3.2.1. Robot Preparation (Fig. 1)

1. Load the following materials and reagents in the appropriate positions on the robot
   - Five 8-tube thermo-strips on the PCR plate (row 1–5, each sample/tube, 5 tubes/run).
   - Pipette tips on the tip racks.
   - Small radioimmunoassay tubes into small reagent reservoir (S1 & S0). Add 15 µL acetonitrile to each tube on S1 and 16 µL magnetic beads to each tube on S0 (*see* **Note 7**).
   - Add water to the large reagent reservoir for tip wash.
   - Add Wash Solution into medium reagent reservoir 1.
   - Add Binding Solution into medium reagent reservoir 2.
   - Add water into medium reagent reservoir 3.
   - Attach a used pipette tip bag to the tip discard.
2. Load 15 µL serum into a thermo-strip tube in row 1 (8 serum samples/strip).
3. Turn on the robot and computer, load the robot software, and initiate the program.

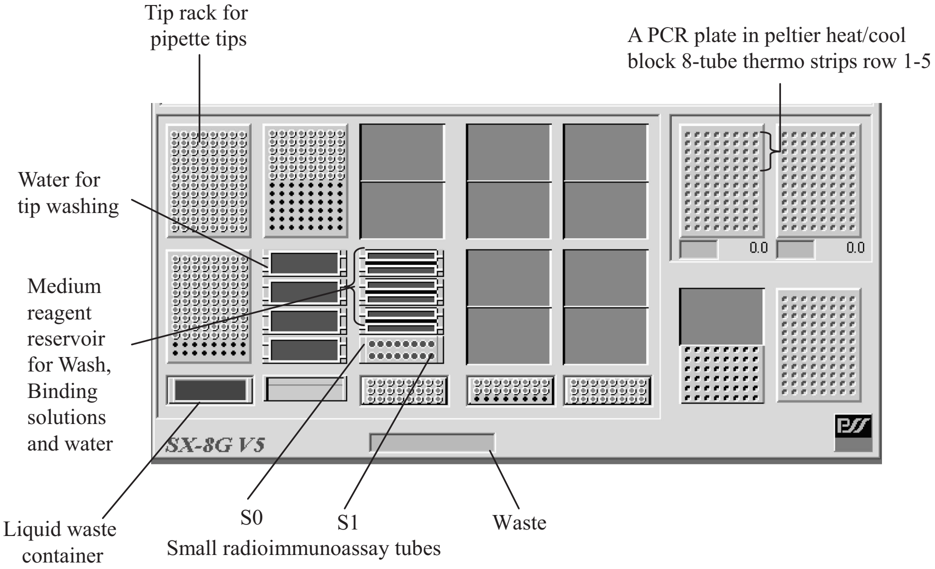
Tip rack for pipette tips
A PCR plate in peltier heat/cool block 8-tube thermo strips row 1-5
Water for tip washing
Medium reagent reservoir for Wash, Binding solutions and water
0.0
0.0
SX-8G V5
PSS
Liquid waste container
S0
S1
Small radioimmunoassay tubes
Waste

Fig. 1. Schematic of the robot.

3.2.2. Automated Steps

1. Robot picks up 10 µl water and transfers to tubes in Row 2.
2. Robot picks up 10 µl acetonitrile from tubes in S1 and transfers to the same tubes in Row 2 to generate 50% aqueous acetonitrile; the solvent is mixed three times by pipetting up and down.
3. Tips are rinsed once with water.
4. Robot picks up 200 µl Wash Solution and distributes 100ul each into tubes in Row 3 and Row 4 (*see* **Subheading 2.2**) (*see* **Note 8**).
5. Robot picks up 100ul Wash Solution and dispenses into tubes in Row 5.
6. Tips are rinsed once in water.
7. Robot picks up 35ul binding solution and mixes thoroughly with sera in Row 1.
8. The tips are changed.
9. Robot picks up 15 µL beads from each tube on S0 and mixes thoroughly with diluted serum; the sample is incubated for 2 min at room temperature (*see* **Note 9**).
10. Beads are captured by the magnet and the supernatant is discarded.
11. Beads are taken to the 8-tube thermo strip on Row 3–5 and washed in wash solution; beads are captured by the magnet and the supernatant is discarded.
12. Proteins/peptides are eluted from the beads at tubes in Row 2 with 20ul of 50% acetonitrile/water.
13. Beads are captured and discarded.
14. Take the strip out of the robot, place a cover on it, and spin briefly in a tabletop centrifuge.
15. Remove stray beads manually with the MagnetSeparator if needed.
16. Transfer eluates into Microcon filters for ultrafiltration.

3.3. Ultrafiltration

1. Pipette 150 µL of water into each assembled 30kDa filter (*see* **Note 10**).
2. Wash filter 5 min at 10,000 g and 10°C in a refrigerated centrifuge; repeat 3 times for a total of four washes.
3. Pipette 20 µL of dH_2O to the washed Microcon filters.
4. Add 20 µL MB eluate to the washed Microcon filters (final concentration 25% acetonitrile).
5. Spin for 10 min at 10,000 g and 10°C in a centrifuge (*see* **Note 11**).
6. Concentrate ultrafiltrate to dryness in a vacuum evaporator (*see* **Note 12**).

3.4. Manual Protocol

1. Add 15 µL of magnetic beads to each tube of the 8-tube thermo strip (*see* **Note 7**).
2. Add 35 µL Binding Solution and 15 uL serum to each tube; pipette up and down for 2 min.
3. Place thermo-strip on a MagnetSeparator; incubate for 1 min to capture the beads.
4. Aspirate supernatant and discard; peptides are bound to the beads.
5. Wash with 100 µL of Wash Solution (transfer strip on the magnet at least 6 times) and discard supernatant.

6. Repeat 2 more times for a total of three washes.
7. Elute peptides from beads using 20 µL of 50% Acetonitrile; incubate for 2 min (*see* **Note 13**).
8. Capture the beads on the magnet and place supernatant into Microcon filter tubes for ultrafiltration.

3.5. MS and Data Analysis

1. Resuspend peptides in 2 µL sample buffer (0.1%TFA/5% acetonitrile/water) (*see* **Note 14**).
2. Spot 2 µL sample on an Anchorchip® target and allow to dry partially (See Note 15).
3. Mix partially dried sample with 1 µL of matrix solution on the target plate and allow to dry completely (*see* **Note 16**).
4. Spot 1 µL of peptide calibration standard and mix on the target with 1 µL matrix then dry completely.
5. Analyze the plate using an Ultraflex MALDI TOF/TOF mass spectrometer (Bruker Daltonics) equipped with pulsed ion extraction ion source. Ionization is achieved by irradiation with nitrogen laser ($\lambda = 337$ nm) operating at 20 Hz. Ions are accelerated at +19 kV with an 80 ns of pulsed ion extraction delay. Each spectrum is detected in linear positive mode at m/z 800–10,000 and is externally calibrated using the Peptide calibration standards (Bruker Daltonics) (*see* **Notes 17** and **18**).
6. Mass spectra are exported using the Flex Analysis software (Bruker Daltonics); raw data are converted to text files for further analysis.
7. Data analysis is carried out as described in the following chapter.
8. **Fig. 2** shows the mass spectrum of a serum sample analyzed using the protocol described above.

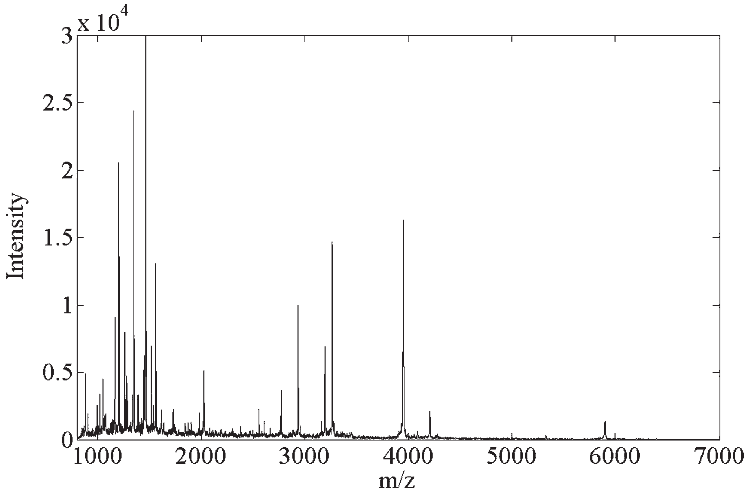

Fig. 2. MALDI-TOF mass spectrum of the C8 magnetic bead-ultrafiltration enriched serum.

4. Notes

1. Manual processing is possible with a skilled technician handling approximately 30 samples in a day. The amount of pipetting at this scale is substantial and difficult to maintain reproducibly over time. The manual handling is described in **Subheading 3.4.**
2. Dissolve CHCA at 5 mg/ml in 50% acetonitrile, aliquot 1mg/vial (0.2 ml), vacuum dry and desiccate at −20°C. For experiments, dissolve fresh in 50% acetonitrile (0.15 ml of water and 0.15 ml of acetonitrile) at a final concentration of 3.3 mg/ml. Protect matrix from exposure to daylight at all times.
3. Control time of clotting as closely as possible. Differences in clotting time may affect results. Follow SOP for hazardous biological materials.
4. Be careful not to disturb the interface between serum and red blood cell clot when aspirating serum. If the interface is disturbed, repeat the centrifugation step.
5. A. We use aliquots of approximately 1 ml further divided at first thaw into 50 μl subaliquots.
   B: It is advisable to keep track of the time from collection to centrifugation and of any lag between centrifugation and freezing.
   C: Avoid multiple freeze-thaw cycles. Freeze-thaw cycles of serum samples can cause degradation of peptides and consequently changes in the mass spectra.
6. Other convenient automation system can be used. Manual processing flow can be also used as described in **Subheading 3.4.**
7. Shake magnetic beads thoroughly to form a homogeneous suspension before transfer to the tubes. Beads are stable for several hours after shaking.
8. The Dynabeads RPC 18 (Invitrogen) can be used as an alternative. The Dynabeads are supplied in 20% ethanol. Make sure to recondition beads in 0.1% TFA before applying samples. 0.1% TFA in water can be used for wash solution, and for binding solution.
9. Pipette gently to avoid making air bubbles.
10. Be careful not to touch the membrane with the tip!!
11. Samples can spin at 4°C but we observe an occasional freezing of the sample at this temperature which could damage the filter membrane.
12. Dried peptides may be stored for several weeks desiccated at −80°C.
13. Disrupt the beads with the tip and pipette up and down, but try to avoid formation of bubbles.
14. Pipette at least 1 min up and down and swirl around the bottom to dissolve the samples well.

15. The plate surface can be slightly touched with the tip but wear gloves to handle the plate. Wait until the spot shrinks to approximately 25% of its original size (about 10 min), but do not dry the spot completely.
16. The spot is dry when yellowish (from the side) and not shiny (from the top). If continuously shiny, add 0.5 µL of matrix. The spot can also be dried in vacuum.
17. For identification of the peptides of interest by sequencing, the reflector mode of the MALDI-TOF-TOF instrument is used.
18. The C8 enriched peptides can be analyzed on other MALDI-TOF instruments such as ABI 4800 Mass Analyzer (Applied Biosystems, Foster City, CA). The 4800 MALDI TOF/TOF TM Analyzer is used in positive ion mode in both reflector and MSMS acquisitions with laser repetition rate at 200 Hz. In MSMS mode, 2 kV collision energy (with CID gas on) is used to fragment the peptides.

Acknowledgment

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Computational Methods for Analysis of MALDI-TOF Spectra to Discover Peptide Serum Biomarkers

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1. Introduction

The characterization of peptides in serum and plasma by mass spectrometry (MS) is one of the promising strategies for biomarker discovery ***(1,2).*** In addition to careful experimental design, improved mass spectrometric technology and sample preparation methods, innovative computational methods are needed to facilitate data interpretation and extract useful information from high-dimensional MS data. In this chapter, we describe computational methods for analysis of raw mass spectra generated by MALDI-TOF/TOF instrument (Bruker Daltonics, Billerica, MA) and exported as text files by the Flex Analysis software. We bin the text files using 'Protein Group Report Generator 1.6', which is a Visual Basic 6 application, and import the binned data into MATLAB, where we apply spectral preprocessing methods as well as statistical and machine learning methods to extract candidate peptide biomarkers.

The purpose of data preprocessing is to correct intensity and m/z values in order to: (1) reduce noise, (2) reduce amount of data, and (3) make the spectra comparable to each other. For example, binning reduces the dimension of a spectrum by grouping intensity measurements at adjacent m/z values into bins. Its aim is to preserve raw data information while performing a dimensional reduction for subsequent processing and mining phases. Smoothing is a process by which data points are averaged with their neighbors as in a time-series of data. Its main aim is to reduce noise, i.e., to increase signal to noise ratio. Baseline correction flattens the base profile of a spectrum to minimize the impact of varying baseline caused by the chemical noise in the matrix or by ion overloading; drifting baseline introduces serious distortion of ion intensities

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without adequate correction. Normalization reduces systematic variation that may be caused by varying amounts of protein, sample degradation over time, or variation in the sensitivity of the MS ion detector. Normalization enables the comparison of different samples since the absolute peaks of different spectra could be incomparable. Peak detection deals with the identification of peaks that display a reasonable intensity compared to those that may be just noise. Hence, the main task of peak detection is separating real peaks (e.g. corresponding to peptides) from peaks representing noise. Although this task can be done visually by mass spectrometry experts, algorithms that do not require human intervention are needed for rapid and repeatable quantitative processing of spectra that often contain hundreds of discrete peaks. The simple peak finding (SPF) provides the locations of all local maximum peaks and their associated left-hand and right-hand bases ***(3)*.** Peak calibration allows correction of drifts that do not reflect any real sample variation. Without peak calibration, the same peak (e.g. the same peptide) can have different m/z values across samples. To allow an easy and effective comparison of different spectra, peak alignment methods find a common set of peak locations (i.e. m/z values) in a set of spectra, in such a way that all spectra have common m/z values for the same biological entities. For example, dynamic programming can be used to align peaks across spectra ***(4)*.** However, this approach and other peak detection tools such as Proteome Quest (Correlogics Systems, Bethesda, MD) deal with exact mass points (single peak). Hence, they do not address the existence of isotopes representing the same peptide, where the same peptide could have multiple peaks. It is important to take into consideration that each detected m/z value is affected by noise and isotopes causing the presence of a window in which the m/z ratio can be shifted. A window is defined here to indicate the range of potential m/z shifting for each peak location ***(5)*.** To automate the creation of windows, neighboring peaks are coalesced, if they fall within a pre-specified mass separation ***(3)*.** A mass separation cutoff is determined based on the instrument's tolerance.

Finally, peaks that best discriminate the subjects within a subgroup are selected via a feature selection algorithm. In mass spectral data analysis, the peak dimensionality is usually larger than or comparable to the sample size. This makes many standard pattern classification algorithms fail to address the high risk of overfitting. Thus, there is an algorithmic need for peak selection in addition to the biological need of discovering a manageable set of key disease biomarkers ***(6)***. A commonly used approach in peak selection is to apply statistical analyses such as t-test and weighting factor, employed in the weighted voting algorithm ***(7)*** that recognize differentially abundant peaks between two groups with multiple subjects. These peaks are then used as inputs to a pattern classification algorithm such as support vector machine (SVM). This approach has the following limitations: (a) it selects peaks based on "relevance criterion", not on the basis of their "usefulness" (i.e., prediction capability); (b) redundant peaks can exist; and (c) peaks that have strong discriminant power jointly, but are weak individually are ignored. The SVM recursive feature elimination (SVM-RFE) algorithm recursively classifies samples with SVM and select features according to their SVM weights ***(8).*** Benefiting from the good performance of SVMs in high dimensional gene-expression data, SVM-RFE is often considered as one of the best feature selection algorithms in the literature. SVM-RFE ranks the features once using all samples, and uses the top ranked features in the subsequent cross-validation. This will generate a biased estimation of errors and limits the search space by allowing only the top ranked features as candidate features. The goal is to search for a manageable combination of useful features from the entire set of peaks. However, due to the large number of peaks, a systematic method is required to select the best combination of peaks without examining all possible combinations. Stochastic global optimization methods such as genetic algorithms (GAs), simulated annealing, and swarm intelligence (SI) methods are ideal candidates for selecting features from a high-dimensional search space. The recent release of ClinProTools™ uses GAs to determine features for SVM classifiers. In this chapter, we provide codes that can be used to select the optimal peak set by combining SVM with a special type of SI method, ant colony optimization (ACO). ACO allows the integration of features selected on the basis of both "relevance" and "usefulness" criteria ***(9)***.

This chapter is organized as follows: Section 2 lists the software tools needed for MALDI-TOF MS data analysis including codes written by the authors of this chapter. Section 3 highlights the methodologies for spectral preprocessing and peak selection. Also, this section outlines the procedure to run the codes, in which the methodologies are implemented. Finally, **Subheading 4** provides additional notes and alternative procedures.

2. Materials

2.1. FlexAnalysis Software

FlexAnalysis software is provided by Bruker Daltonics (Billerica, MA). Raw mass spectra generated by MALDI-TOF/TOF instrument can be viewed and saved using this software.

2.2. Binning Software Tool

1. Protein Group Report Generator 1.6 (PGRG) (http://microarray.georgetown.edu/files/msdat.zip)
2. VB6 Run-time and VB6 Service Pack available at Microsoft website.

2.3. MATLAB Tools

1. MATLAB software (Windows version)
2. MATLAB Bioinformatics Toolbox

3. MATLAB Statistics Toolbox
4. OSU SVM Toolbox for MATLAB (http://sourceforge.net/projects/svm/)
5. MATLAB codes (http://microarray.georgetown.edu/files/msdat.zip). These codes include m-files the authors of this manuscript wrote and additional m-files from The Cromwell Package (http://bioinformatics.mdanderson.org/cromwell.html) and SVM-RFE files from (http://www.bo.infn.it/ masotti/software.html)

3. Methods

Figure 1 illustrates our methodology for peak selection. The spectra in the labeled set are used for peak selection. The resulting peaks and the associated SVM classifier is used to predict the disease state of the spectra in the blinded set. Spectral preprocessing (i.e., binning, baseline correction, normalization, peak detection, and peak calibration) and peak selection are performed on the labeled set by subjecting the entire process to cross-validation.

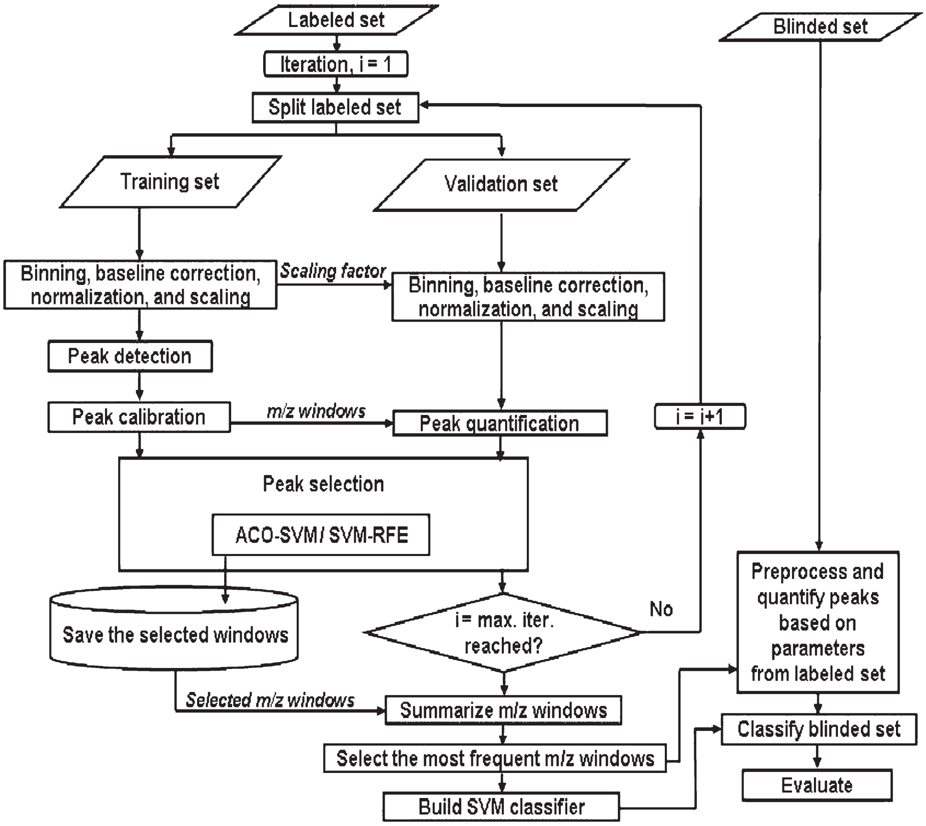
Labeled set
Blinded set
Iteration, i = 1
Split labeled set
Training set
Validation set
Binning, baseline correction, normalization, and scaling
Scaling factor
Binning, baseline correction, normalization, and scaling
Peak detection
Peak calibration
m/z windows
Peak quantification
i = i+1
Peak selection
ACO-SVM / SVM-RFE
Preprocess and quantify peaks based on parameters from labeled set
Save the selected windows
i = max. iter. reached?
No
Selected m/z windows
Summarize m/z windows
Classify blinded set
Select the most frequent m/z windows
Evaluate
Build SVM classifier

Fig. 1. Methodology for peak detection.

As illustrated in **Fig. 1,** the spectra in the labeled set are split into training and validation sets. The performance of the peaks selected from the training spectra are evaluated via validation spectra to guide the search for the optimal peak set. In the following, we describe briefly the specific methodologies we implemented for spectral preprocessing and peak selection.

3.1. Spectral Preprocessing

Binning reduces the dimension of a spectrum by grouping intensity measurements at adjacent m/z values into bins. Its aim is to preserve raw data information while performing a dimensional reduction for subsequent processing and mining phases

We estimate the baseline of a binned spectrum by obtaining the minimum value within a shifting window size of 50 bins and a step size of 50 bins of each spectrum. Spline approximation is applied to regress the varying baseline. The regressed baseline is smoothed using the lowess smoothing method. The resulting baseline is subtracted from the spectrum.

Each spectrum is normalized by dividing by its total ion current. After scaling the peak intensities of the normalized training spectra to an overall maximum intensity of 100, local maximum peaks above a specified threshold are identified and peaks that fall within a prespecified mass separation are coalesced into a single m/z window to account for drift in m/z location and to represent isotopic clusters by a single peak. The maximum intensity in each window is used as the variable of interest.

3.2. Peak Selection

We apply the hybrid ACO-SVM algorithm to search for a peak set that consists of a pre-specified number of peaks. ACO-SVM selects a peak set on the basis of its ability to distinguish the case and control spectra in the validation set. Note that the spectra in the validation set are not involved in the spectral preprocessing. They are binned, baseline corrected, normalized, and scaled on the basis of the parameter used to preprocess the spectra in the training set. These parameters include scaling factor that standardizes the peaks in the training set to have a maximum of 100. The peaks in the validation set are quantified at the selected m/z windows and are presented to SVM classifier previously trained using the peaks from the training set. The performance of the SVM classifier in predicting the disease state of the subjects in the validation set is used by ACO-SVM to guide its search for the optimal peak set.

Alternatively, the user can choose the SVM-RFE algorithm, which uses the labeled set to determine the most useful set of peaks.

The above steps (spectral preprocessing and peak selection) are repeated multiple times by randomly splitting the labeled spectra into training and validation sets with resubstitution. The peaks selected in multiple iterations (I = 1,2,…) are summarized by merging overlapping m/z windows to determine the most frequently selected m/z windows. Note that the number of peaks detected and the size of the m/z windows could vary due to the change in the population set at each iteration.

To evaluate the peak selection process, we quantify the peak intensities at the m/z windows of the final peak set in both the labeled and blinded sets. Note that the blinded set is not used during the entire peak selection process, thus it serves as an independent set to evaluate the generalization capability of the selected peaks. Alternatively, if the disease state of the spectra in the blinded set is unknown, an SVM classifier built via the labeled set will be used for prediction. The spectra in the blinded set are binned, baseline corrected, normalized, and scaled on the basis of parameters used to preprocess the spectra in the labeled set.

3.3. Procedure

1. Place the provided *PGRG.exe* in any directory and create the following folders: “INPUT_GRP1”, “INPUT_GRP2”, and “OUTPUT”
2. Place input files (i.e., .TXT files from FlexAnalysis software) into either INPUT_GRP1 or INPUT_GRP2. Alternatively, split the TXT files into two phenotypic groups and place them in the two folders.
3. Run the PGRG.exe
4. Provide values for all the form fields

Note: Choose the CSV option for Output file. The Output File name should not contain characters like / : etc.

5. Choose either “Sum Intensity Values per Reported ID” or “Calculate Average Intensity per Reported ID.”
6. Press the Generate Report button.

The application generates an Output File in the OUTPUT folder. PGRG uses data from all the TXT files stored in the INPUT_GRP1 or INPUT_GRP2 or both directories to generate the report. If only one of the INPUT folders contains input files, then a simple report will be generated. If both input folders contain input files, the generated report contains five more columns for group comparison purposes.

7. Copy the EXCEL file that has the binned data into a folder where the MATLAB files are copied.
8. Start MATLAB
9. Run the m-file *ProteomicAnalysis.m*, which will prompt you to provide the following information sequentially:
    - 9.1 Enter the name of the EXCEL file which has the training data. After it reads the data into MATLAB.

9.2 Choose a peak selection method; the options are “SVM-RFE” (default) and ACO-SVM.
9.3 Enter the number of features to be selected. The default is set to three. It is advised to use at least three features.
9.4 Enter the number of times (iterations) the reshuffling algorithm is to be done. The code will run through the baseline correction and normalization process, peak detection, and peak selection process.
9.5 Select the desired number of peaks. (Note that if the reshuffling is done only once, the number of peaks will be equal to the number entered in Procedure 9.3. However, for multiple iterations, a frequency plot will be provided to assist the user to select the number of desired peaks).
9.6 Enter a filename you wish to save the results as a MAT file. The result includes parameters used for normalization, along with the normalized train data, the SVM classifier built for the training data and the selected m/z windows. These will be saved as a MAT file in the name you have provided when prompted for.

10. To predict the disease state of spectra in a blinded set, bin the blinded spectra following steps 1 to 6. Run the M-file *PredictResults.m*. The m-file will prompt you to provide the following information sequentially:

10.1 Enter the name of the EXCEL file which has the blinded data
10.2 Enter the name of the saved MAT file (which has parameters used to analyze the labeled set) from the labeled data.

The code will perform baseline correction and normalization in the blinded dataset and then quantify the peaks/windows based on the selected peaks from the labeled data. It also uses the saved SVM classifier information and then gives you labels for the blinded dataset.

11. As an alternative to step 10, the m-file *SummarizeResults.m* allows you to choose a new set of summarized peaks from the previously trained spectra. Running this code prompts you to provide the following information sequentially:

11.1 Enter the name of the EXCEL file which has the binned blinded data
11.2 Enter the name of the saved MAT file that has parameters used by the labeled set. If the labeled set was shuffled more than once, a frequency plot will be displayed for the summarized peaks.
11.3 Choose the number of peaks based on the frequency plot. These peaks will be used to design an SVM classifier which will then predict the labels for the blinded set.

4. Notes

1. As pointed out in Procedures 2 and 6, our binning tool can read .TXT files generated by the FlexAnalysis software either from a single input folder or two folders. If a single input folder is used, the output file will have one

column per subject. If the files are placed into two input folder, then the output file will provide the following additional five columns for group comparison purposes:

[GRP1] "Avrg Intensity" = an Average GRP1 intensity per row
[GRP1] "Val Count" = number of GRP1 intensity values per row
[GRP2] "Avrg Intensity" = an Average GRP2 intensity per row
[GRP2] "Val Count" = number of GRP2 intensity values per row
"GRP |Dif|" = the absolute difference between the [GRP1] and [GRP2]

2. If 'ACO-SVM' is chosen in Procedure 9.2, the algorithm will need a long time to perform the feature selection for multiple generations. The number of ants in this algorithm is set by default to 50 and the number of generations is set to 500. To change these options, the user needs to edit the m-files *PeakSelection_ACOSVM.m* and *peak_ACO_S2N.m*. However, the default peak selection algorithm in Procedure 9.2 (SVM-RFE) requires relatively less computational time.
3. If the feature selection algorithm is run only once (i.e., if number of reshuffling is set to 1 in Procedure 9.4), then frequency plot will not be provided. We suggest that the user performs multiple reshuffling (10–100 iterations) to determine the most frequently selected peaks.
4. If the feature selection algorithm is run for multiple iterations by reshuffling the labeled spectra, then a summarized set of peaks will be provided in Procedure 9.5. The summarization is needed because, when the samples are reshuffled, the number of peaks found for each run and the width of each peak may vary. Summarization allows us to merge overlapping peaks and to provide a frequency plot of the number of occurrence of each summarized peak in multiple iterations. The number of peaks needed for classification of a blinded set can be chosen based on a frequency plot, which assists users to estimate the optimal number of peaks. The frequency plot presents a bar plot with the number of occurrences versus the peaks sorted in the order of decreasing frequency. A commonly used approach is to select all peaks starting from the first until the frequency curve becomes flat (i.e. the change in frequency becomes low).
5. The MAT file saved following Procedure 9.6 consists of various training parameters including scaling factor, selected peaks, normalized labeled spectra, and an SVM classifier built based on the spectra in the labeled set. If multiple iterations are used in Procedure 9.4, the SVM classifier uses the peaks selected by the user on the basis of the frequency plot (Procedure 9.5). Otherwise, the number of peaks will be equal to the value entered in Procedure 9.3. The training parameters are used by *PredictResults.m* to

predict the disease state of the spectra in the blinded set. If the user wants to change the number of peaks without rerunning the peak selection process, previously saved training parameters from Procedure 9.6 can be used by *SummarizeResults.m* (Procedure 11) to display the frequency plot and to allow the selection of the number of peaks needed. Once the number of peaks are selected (Procedure 11.3), *SummarizeResults.m* predicts the disease state of the spectra in the blinded set.

Acknowledgments

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Part V

POSTTRANSLATIONAL MODIFICATIONS

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Simple Tools for Complex *N*-Glycan Analysis

Anne-Catherine Fitchette, Meriem Benchabane,
Thomas Paccalet, Loïc Faye, and Véronique Gomord

1. Introduction

Glycosylation is the most widespread post-translational modification encountered on secreted glycoproteins. In eukaryotes, glycosylation can be of two types: *N*- or *O*-glycosylation, depending on the linkage involved between the oligosaccharide and the protein backbone. The latter will not be detailed here.

N-glycosylation occurs exclusively on proteins that enter the secretory pathway of eukaryotic cells. It starts in the endoplasmic reticulum (ER) with the cotranslational transfer of an oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, onto specific asparagine residues constitutive of *N*-glycosylation sequences present on the nascent protein (Asn-*X*-Ser/Thr, where *X* can be any amino acid except proline). Then, processing of the $Glc_3Man_9GlcNAc_2$ precursor occurs along the secretory pathway as the glycoprotein moves from the ER through the Golgi apparatus to its final destination. Glycosidases and glycosyltransferases convert successively the oligosaccharide precursor into high-mannose-type *N*-glycans, ranging from $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$, and to complex-type *N*-glycans (**Fig. 1**). This maturation pathway is common to most eukaryotic cells, including plant and human cells, up to the $Man_5GlcNAc_2$ structure (**Fig. 1**). Contrastingly, complex-type *N*-glycans differ according to the cell system in which they are matured. In plants, for example, the proximal GlcNAc of the core can be substituted with an α1,3-fucose, instead of an α1,6-fucose for human *N*-glycans. The β-mannose of the core can be substituted with a β1,2-xylose or a β1,4-GlcNAc in plants and humans, respectively. Moreover, glycan antennae are composed of a terminal β1,3-galactose and an α1,4-fucose linked to the terminal GlcNAc, forming the Lewis a structure, in plants (**Fig. 1**) *(1)*. In mammals, especially

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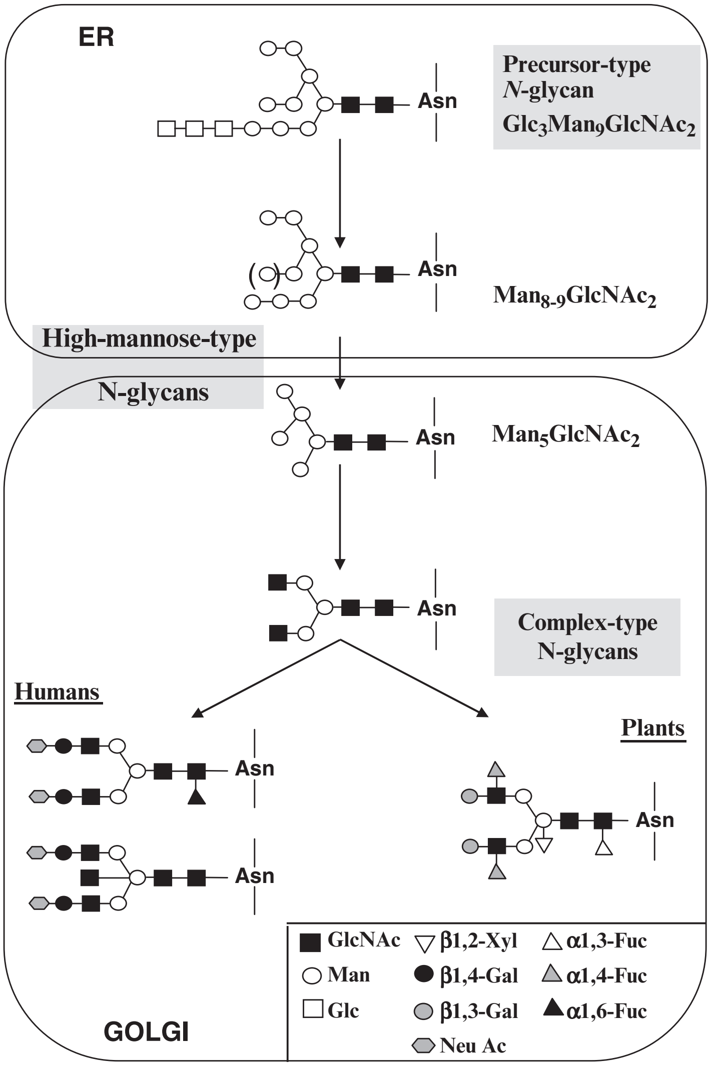
ER
Precursor-type
N-glycan
$Glc_3Man_9GlcNAc_2$
Asn
( )
$Man_{8-9}GlcNAc_2$
High-mannose-type
N-glycans
$Man_5GlcNAc_2$
Complex-type
N-glycans
Humans
Plants
GlcNAc
Man
Glc
β1,2-Xyl
β1,4-Gal
β1,3-Gal
Neu Ac
α1,3-Fuc
α1,4-Fuc
α1,6-Fuc
GOLGI

Fig. 1. *N*-glycan maturation pathway in humans and plants. (GlcNAc: N-acetylglucosamine, Man: mannose, Glc: glucose, Xyl: xylose, Gal: galactose, NeuAc: N-acetyl neuraminic acid, Fuc: fucose).

humans, terminal GlcNAc are substituted with β1,4-galactose to which neuraminic acid can be linked (**Fig. 1**).

This discrepancy between mammalian and plant complex *N*-glycans raises problems when a recombinant plant glycoprotein is to be used as a pharmaceutical in human. Indeed, the plant β1,2-xylose and the α1,3-fucose have been shown to be immunogenic in all laboratory mammals, with the exception of the

BALB/c mouse. The presence of xylose and fucose on a plant pharmaceutical will render this therapeutical protein insecure for injection to humans. Moreover, the differences existing between plant and human complex *N*-glycan structures can provoke a loss in biological activity and/or half-life of the plant pharmaceutical, once administrated. To circumvent these problems, several *in vitro* or *in vivo* strategies are being developped currently to humanize the *N*-glycans from plant pharmaceuticals (for recent review, see ***2***). In any case, *N*-glycosylation studies of a mammalian/human protein and its plant-expressed counterpart are generally undertaken to highlight the differences between the glycosylation status of these proteins.

In the present chapter, the authors propose simple methodologies to define whether and how a protein, expressed in mammal or plant, is glycosylated.

2. Materials

2.1. Is My Protein a Glycoprotein?

1. DIG Glycan Detection Kit (Roche Applied Science, Mannheim, Germany) (store at 2–8°C).

2.2. How Is My Protein Glycosylated?

2.2.1. Detection of N-Glycans by Western Blotting

2.2.1.1. AFFINODETECTION WITH LECTINS

1. TTBS buffer: 20 m*M* Tris-HCl, pH 7.4, containing 0.5 *M* NaCl and 0.1% Tween-20.
2. Lectin-biotin: GNA (*Galanthus nivalis* agglutinin)-biotin (Vector Labs/Abcys, France) (store at 2–8°C), (*Triticum vulgaris* agglutinin)-biotin (Sigma-Aldrich, Lyon, France) (store at 2–8°C), MAL II (*Maackia amurensis* lectin II)-biotin (Vector Labs/Abcys, France) (store at 2–8°C), RCA I or RCA_{120} (*Ricinus communis* agglutinin I)-biotin (Vector Labs/Abcys, France) (store at 2–8°C), SNA (*Sambucus nigra* agglutinin)-biotin (Vector Labs/Abcys, France) (store at 2–8°C).
3. Streptavidin-peroxidase conjugate (Amersham Biosciences/GE Healthcare, Uppsala, Sweden) (store at 2–8°C).
4. TBS buffer: 20 m*M* Tris-HCl, pH 7.4, containing 0.5 *M* NaCl.
5. 4-Chloro-1-naphtol (Bio-Rad, Hercules, CA) (store at −20°C).
6. 37% H_2O_2.
7. Ribonuclease B from bovine pancreas (Sigma-Aldrich) (store at 2–8°C).
8. Methyl α-D-mannopyranoside (Sigma-Aldrich) (store at room temperature).
9. Ovalbumin (Sigma-Aldrich) (store at 2–8°C).
10. TTBS* buffer: 20 m*M* Tris-HCl, pH 7.4, containing 0.5 *M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 0.1% Tween-20.
11 Concanavalin A (Sigma-Aldrich) (store at 5–8°C).
12 Horseradish peroxidase (HRP, Sigma-Aldrich) (store at 2–8°C).
13 Soybean agglutinin (Sigma-Aldrich) (store at 2–8°C).
14 Fetuin from fetal calf serum (Sigma-Aldrich) (store at 2–8°C).

15 Glycophorin from blood type B negative (Sigma-Aldrich) (store at 2–8°C).
16 Asialofetuin from fetal calf serum (Sigma-Aldrich) (store at 2–8°C).

2.2.1.2. IMMUNODETECTION WITH SPECIFIC ANTIBODIES

1. TBS buffer: 20 m*M* Tris-HCl, pH 7.4, containing 0.5 *M* NaCl.
2. Gelatin (Bio-Rad).
3. Anti-HRP rabbit immunserum (Sigma-Aldrich) (store at 2–8°C).
4. Anti-*Apis mellifera* venom (honeybee venom; Sigma-Aldrich) (store at 2–8°C).
5. Mouse anti-human Lewis a monoclonal antibody (Calbiochem, Meudon, France) (store at −20°C).
6. TTBS buffer: 20 m*M* Tris-HCl, pH 7.4, containing 0.5 *M* NaCl and 0.1% Tween-20.
7. HRP-conjugated goat antibodies directed at mouse polyvalent immunoglobulins (Sigma-Aldrich) (store at −20°C).
8. HRP-conjugated goat antibodies directed at rabbit immunoglobulins (Bio-Rad) (store at 2–8°C).
9. Phospholipase A2 from honeybee venom (Sigma-Aldrich) (store at 2–8°C).
10. Bean phytohemagglutinin L (PHA-L, Sigma-Aldrich) (store at 2–8°C).

2.2.2. Analysis of the Protein after Enzymatic Glycan Release

1. 1% Sodium dodecyl sulfate (SDS).
2. 150 m*M* Sodium acetate buffer, pH 5.7.
3. Endo H: endoglycosidase H from *Streptomyces plicatus,* recombinant from *E. coli* (Roche Applied Science) (store at 2–8°C; *see* **Note 1**).
4. 0.1 *M* Tris-HCl buffer, pH 7.5, containing 0.1% SDS.
5. 0.1 *M* Tris-HCl buffer, pH 7.5, containing 0.5% Nonidet P40.
6. Nonidet P40 (called now IGEPAL; Sigma-Aldrich).
7. PNGase F: peptide-*N*-glycosidase F from *Flavobacterium meningosepticum* (Roche Applied Science) (store between −15°C and −25°C).
8. 10 m*M* HCl solution, pH 2.2.

2.2.3. In Vitro Enzymatic N-Glycan Sequencing Followed by SDS-Page Analysis

2.2.3.1. IN VITRO SEQUENCING BY EXOGLYCOSIDASES

1. *Arthrobacter ureafasciens* neuraminidase (from EY Laboratories, Coger, France) (store at −20°C).
2. 90 m*M* sodium phosphate, pH 5.0.
3. β-galactosidase from *Aspergillus oryzae* (Sigma Aldrich) (store at −20°C).
4. SP-Sepharose Fast Flow (Amersham Biosciences/GE Healthcare) (store at 4°C).
5. Sephacryl S200 HR (Amersham Biosciences/GE Healthcare) (store at 4°C).
6. pNP-β-Gal: 2-nitrophenyl β-D-galactopyranoside (Sigma-Aldrich) (store at −20°C).
7. 10m*M* sodium acetate, pH 4.6.
8. 0.5 *M* NaCl.
9. 50 m*M* Tris-HCl, pH 7.5, containing 0.1 *M* NaCl.
10. 50 mM sodium acetate, pH 5.0.

2.2.3.2. In Vitro Modification with Glycosyltransferases

1. β1,4-Galactosyltransferase from bovine milk (Calbiochem) (store at −20°C).
2. UDP-Galactose (uridine 5′-diphosphogalactose disodium salt) (Sigma-Aldrich) (store at −20°C).
3. 100 m*M* Sodium cacodylate, pH 6.4, containing 5 m*M* UDP-galactose and 5 mM $MnCl_2$.

3. Methods

3.1. Is My Protein a Glycoprotein?

To determine if a protein can be *N*-glycosylated, a first point to check is the presence of potential *N*-glycosylation sites (Asn-*X*-Ser/Thr) within its sequence. This can be performed using the available programs Prosite Scan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html) or NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/). The absence of the Asn-X-Ser/Thr motif within the sequence of a protein is insurance that it is not *N*-glycosylated. At the opposite, the presence of one or more of those motifs does not imply that the protein is a glycoprotein. This should be checked by the methods proposed hereunder.

Only one approach is available to answer the question globally: is my protein a glycoprotein? It necessitates the use of a general sugar detection kit that allows the detection and quantification of glycoproteins on blot. All the reagents necessary for such a determination are provided in a detection kit, the DIG Glycan Detection Kit commercialized by Roche Applied Science, which also includes the manufacturer's instructions. It should be noted that the only information the experimenter will get using this kit is that the proteins do bear at least one oligosaccharide moiety. No information will be provided about the type of glycan involved or its linkage to the protein backbone.

3.2. How Is My Protein Glycosylated?

Several strategies can be initiated to determine the nature of the glycan borne by a glycoprotein. The first one involves the use of probes specific for glycoprotein *N*-glycan moieties in Western blotting experiments. This strategy does not require any glycan cleavage or separation (*see* **Subheading 3.2.1.**). Alternatively, the *N*-glycan can be selectively cleaved from the purified glycoprotein by enzymatic treatment. The glycoprotein is then analyzed by electrophoresis prior to and after deglycosylation (*see* **Subheading 3.2.2**). The same strategy can be initiated on a crude protein extract. The glycoprotein of interest will be immunodetected, after SDS-PAGE migration and Western blotting, by specific antibodies. The removal of *N*-glycans from a glycoprotein induces an increase in its electrophoretic mobility. Roughly, a *N*-linked glycan has a molecular mass between 1,000 and 2,000 Da.

3.2.1. Detection of N-Glycans by Western Blotting

The detection of *N*-glycans borne by glycoproteins can be performed after Western blotting of either 1D or 2D electrophoresis gels. The probes used for this detection are of two types: lectins or antibodies.

3.2.1.1. AFFINODETECTION WITH LECTINS

Lectins are proteins able to bind oligosaccharide moieties specifically. Most lectins have been characterized on the basis of their affinity for mammalian glycoproteins. Only a few of them are available for the detection of plant glycans. They are presented in **Table 1** (*see* **Note 2**).

These lectins are commercially available in a biotinylated form. The recognition event between the lectin and the oligosaccharide is visualized on a blot using a streptavidin-peroxidase conjugate. An exception is the lectin concanavalin A, which is able to bind directly to horseradish peroxidase. The two protocols are detailed here.

The lectin-biotin/streptavidin-peroxidase procedure:

1. Separate the proteins to be studied by 1D or 2D electrophoresis and transfer onto a nitrocellulose membrane.
2. Saturate the blot with TTBS buffer for at least 1 h (*see* **Note 3**).
3. Incubate the blot in TTBS containing the lectin-biotin complex (0.1 mg/20 mL) for 2 h at room temperature.
4. Wash four times in TTBS, for 15 min each.
5. Incubate the blot with a streptavidin-peroxidase conjugate diluted 1:1000 in TTBS for 1 h at room temperature.
6. Wash the blot in TTBS (4 × 15 min) and once in TBS prior to development.
7. Prepare extemporaneously the peroxidase development mixture by dissolving, in a beaker, 30 mg of 4-chloro-1-naphtol in 10 mL cold methanol (−20°C), and mixing in another beaker 30 µL H_2O_2 with 50 mL TBS. Mix the two solutions just prior to pouring onto the membrane, and shake gently to optimize the development reaction.
8. Stop the reaction by discarding the development mixture and rinsing the blot several times with distilled water. Dry the membrane and store between 2 sheets of filter paper.
9. Suggested controls for the experiment are presented in **Note 4**.

The concanavalin A (ConA)-peroxidase procedure (modified from **ref. *3***):

1. Separate the proteins to be studied by 1D or 2D electrophoresis, and transfer onto a nitrocellulose membrane.
2. Saturate the blot for at least 1 h in TTBS at room temperature.
3. Incubate the membrane in TTBS* containing ConA (25 µg/mL) for 2 h at room temperature.
4. Wash the membrane four times for 15 min each in TTBS.

Table 1
Lectins used for *N*- linked glycan detection in Western blotting experiments

Lectin	Specificity	Detected oligosaccharides	Suggested positive controls	Sugar inhibitor	Ref
Concanavalin A from *Canavalia ensiformis*	αD – mannose αD - glucose	- Precursor structures: M9G3 to M9G1 - High-mannose structures: M9 to M5 In plants and animals	Soybean agglutinin Ovalbumin Ribonuclease B	methyl α-D-mannopyranoside	***3***
Galanthus nivalis agglutinin (GNA)	Terminal α1,3-mannose	- High-mannose structures: M8 to M5 In plants and Animals	Ribonuclease B	methyl α-D-mannopyranoside	***22*** ***23***
Wheat germ aggultinin (WGA) from *Triticum aestivum*	Terminal GlcNAc Internal chitobiose units	- All *N*-linked glycans In plants and animals	Ovalbumin	GlcNAc (*see* **Note 17**) Chitotriose Chitobiose	***24***
Ricinus communis agglutinin (RCA I, RCA120)	Terminal Gal α 1,4-GlcNAc	- Complex *N*-glycans devoid of NeuAc (in animals) - *N*-glycans from plants expressing human galactosyltransferase - *N*-glycans remodeled in vitro by neuraminidase or galactosyltransferase	Asialofetuin	Lactose	***25*** ***26; 27***
Maakia amurensis lectin (MAL II)	Terminal NeuAc-α 2,3-Gal– α 1,4-GlcNAc	- Complex *N*-glycans in animals	Fetuin	Lactose	***28***
Sambucus nigra agglutinin (SNA)	Terminal NeuAc-α 2,6-Gal- α1,4-GlcNAc	- Complex *N*-glycans in animals	Glycophorin	Lactose	***28***

5. Incubate the membrane in TTBS containing horseradish peroxidase (HRP, 50 µg/mL) for 1 h at room temperature.
6. Rinse the blot four times for 15 min each in TTBS and once in TBS for 15 min.
7. Develop the peroxidase reaction as described in the lectin-biotin/streptavidin-peroxidase procedure.
8. Controls have to be performed as presented in **Note 4**.

3.2.1.2. IMMUNODETECTION WITH SPECIFIC ANTIBODIES

We have shown that the β1,2-xylose and the α1,3-fucose epitopes of plant *N*-linked complex glycans are highly immunogenic in rabbits ***(4)***. As a consequence, sera prepared against glycoproteins containing complex glycans generally contain antibodies directed at the β1,2-xylose and/or the α1,3-fucose. Some commercially available immunsera present the same characteristics as our homemade probes. For instance, immunsera directed against HRP can be used as probes specific for plant complex *N*-glycans with α1,3-fucose and β1,2-xylose residues. More specific is the immunserum raised against honeybee venom proteins, which can be used as a specific probe for α1,3-fucose-containing plant complex *N*-glycans ***(4)*** (*see* **Note 5**). We also raised in our laboratory rabbit antibodies specific for the terminal antennae of complex *N*-glycans, the trisaccharide Lewis a ***(1)***. Anti-Lewis a monoclonal antibodies are commercially available, although they are very expensive (*see* **Note 6**).

1. Separate the proteins by 1D or 2D electrophoresis and transfer onto a nitrocellulose membrane.
2. Saturate the blot with 3% gelatin prepared in TBS, for at least 1 h at room temperature.
3. Incubate the blot in TBS containing 1% gelatin and the immunserum at a convenient dilution for 2 h at room temperature. We used the commercial immunsera described above in **Subheading 2.2.1.2**. at the following dilutions:
   a. Anti-HRP rabbit immunserum, 1:300.
   b. Anti-honeybee venom rabbit immunserum, 1:200.
   c. Anti-Lewis a mouse monoclonal antibody, 1:100.
4. Wash the blot with TTBS buffer four times for 15 min each.
5. Incubate the blot in TBS containing 1% gelatin and the suitable conjugate, i.e., a goat anti-rabbit IgG conjugate coupled to HRP at a dilution of 1:2000, or HRP conjugated goat antibodies directed at mouse polyvalent immunoglobulins at a dilution of 1:500, for 1 h at room temperature.
6. Wash the blot four times with TTBS for 15 min each and once in TBS prior to development.
7. Develop the peroxidase reaction as described for the lectin-biotin/streptavidin-peroxidase procedure (*see* **Subheading 3.2.1.1.**).
8. For controls, *see* **Notes 7** and **8**.

3.2.2. Analysis of the Protein after Enzymatic Glycan Release

Further information can be obtained by treating the purified glycoprotein with glycosidases. Endoglycosidase H (Endo H) is only able to release high-mannose type *N*-glycans, from plant and mammalian glycoproteins, by hydrolyzing the glycosidic bond between the two GlcNAc residues on the core of the *N*-glycan (**Fig. 2**). Peptide *N*-glycosidases (PNGases) hydrolyze the bond between the Asn of the peptide backbone and the proximal GlcNAc of the oligosaccharide part, for both high-mannose type *N*-glycans and complex type *N*-glycans. However, PNGase F, which is widely used in the analysis of mammalian glycoproteins, is active on high-mannose and complex plant *N*-glycans, except those presenting an α1,3-fucose residue linked to the proximal GlcNAc. PNGase A is able to release all types of plant *N*-glycans (**Fig. 2**), but it is almost only efficient on glycopeptides and necessitates the proteolytic digestion of the glycoprotein prior to deglycosylation (***5, 6***) Therefore, this enzyme cannot be used to deglycosylate a glycoprotein before its electrophoretic analysis (*see* **Notes 9** and **10**). The deglycosylated protein or peptides can be analyzed for 1) an increased electrophoretic mobility, or 2) the loss of glycoprotein reactivity on blots with glycan-specific probes after Endo H or PNGase F treatment.

Deglycosylation with endoglycosidase H:

1. Prior to enzyme deglycosylation with Endo H, denature the purified protein by heating it to 100°C for 5 min in the presence of 1% SDS (w/v).
2. Dilute the sample fivefold with 150 m*M* sodium acetate, pH 5.7, and add then 10 mU Endo H per mg of protein to be analyzed. Incubate the mixture at 37°C for at least 6 h.
3. After the deglycosylation reaction, add an equal volume of twice concentrated electrophoresis sample buffer if the result of the digestion is followed by electrophoresis gel, affino-, or immunodetection (*see* **Subheading 3.2.1.**). The sample can also be desalted and analyzed by mass spectrometry.

Deglycosylation with PNGase F:

1. Dissolve the purified protein to be digested in 0.1 m*M* Tris-HCl, pH 7.5, containing 0.1% SDS.
2. Heat the sample for 5 min at 100°C to denature the protein. Let it cool down at room temperature and add an equal volume of 0.1 m*M* Tris-HCl, pH 7.5, containing 0.5% Nonidet P-40 (*see* **Note 11**).
3. Incubate the sample with PNGase F (1U enzyme for 100 μg protein) at 37°C during 24 h.
4. After digestion, precipitate, by 4 vol of cold ethanol, the deglycosylated protein overnight at −20°C or twice with shorter incubation time.
5. Wash the pellet 2 to 3 times with cold ethanol.

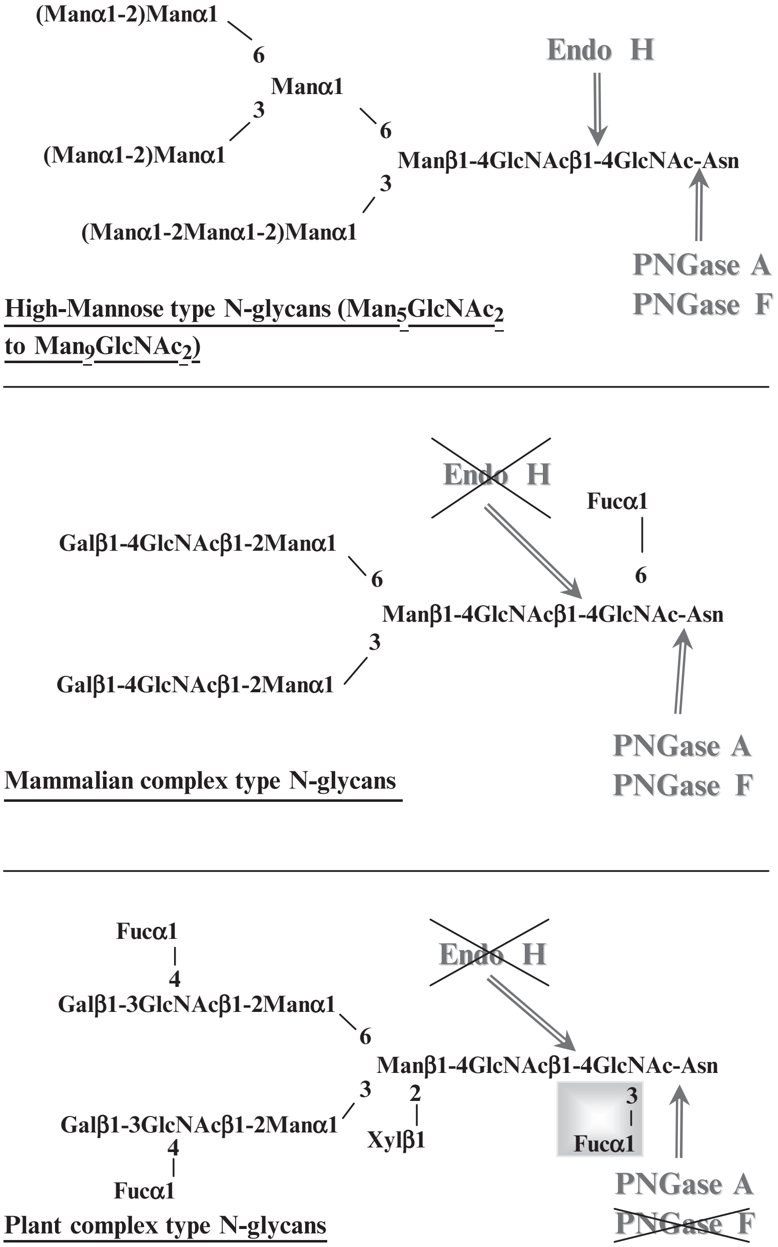
(Manα1-2)Manα1
6
Manα1
3
6
(Manα1-2)Manα1
Endo H
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
3
(Manα1-2Manα1-2)Manα1
PNGase A
PNGase F
High-Mannose type N-glycans (Man5GlcNAc2 to Man9GlcNAc2)
~~Endo H~~
Fucα1
6
Galβ1-4GlcNAcβ1-2Manα1
6
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
3
Galβ1-4GlcNAcβ1-2Manα1
PNGase A
PNGase F
Mammalian complex type N-glycans
Fucα1
4
~~Endo H~~
Galβ1-3GlcNAcβ1-2Manα1
6
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
3
2
3
Galβ1-3GlcNAcβ1-2Manα1
Xylβ1
Fucα1
4
Fucα1
PNGase A
~~PNGase F~~
Plant complex type N-glycans

Fig. 2. Enzymatic deglycosylation of proteins bearing *N*-linked glycans. High-mannose type *N*-glycans can be cleaved by either endoglycosidase H (Endo H), peptide *N*-glycosidase A (PNGase A), or peptide *N*-glycosidase F (PNGase F). Mammalian complex type *N*-glycans can be removed using PNGase A and PNGase F. Plant complex type *N*-glycans can only be released by PNGase A, owing to the presence of α1,3-fucose linked to the core. It should be noted that PNGase A mostly acts on glycopeptides and is not efficient on a complete glycoprotein.

6. Centrifuge the sample. Recover the deglycosylated protein in the pellet and dissolve it in a suitable buffer for gel electrophoresis or mass spectrometry analysis.

3.2.3. In Vitro Enzymatic N-Glycan Sequençing Followed by SDS-Page Analysis

To specify the structure of a *N*-glycan linked to a glycoprotein, exoglycosidases and glycosyltransferases can be used. The formers specifically remove the last sugar residues present at the terminal non-reducing end of the *N*-glycan, while the latters add specifically terminal sugar residues. The resulting modified *N*-glycans are detected on blots with *N*-glycan-specific probes (*see* **Subheading 3.2.1**; *see* **Table 2**).

3.2.3.1. In Vitro Sequencing by Exoglycosidases

Many exoglycosidases can be used to sequence *N*-glycans, illustrated here for two of them. They are neuraminidase from *Arthrobacter ureafasciens* and β1,4-galactosidase from *Aspergillus oryzae*. The former removes any NeuAc present at the non reducing end of *N*-glycans, while the latter cleaves terminal β1,4-Gal (*see* **Table 2**; *see* **Note 12**).

Terminal neuraminic acid enzymatic hydrolysis (*see* **Note 13**):

1. Dissolve 5 µg of protein (*see* **Note 14**) in 20 µL of 90 m*M* sodium phosphate, pH 5.0, and incubate in the presence of 0.5 U neuraminidase for 3 h at 37°C.
2. Dilute then the sample in a suitable electrophoresis buffer prior to SDS-PAGE migration, Western transfer and detection with SNA I and/or RCA_{120} lectins (*see* **Subheading 3.2.1.1**).The loss of terminal NeuAc induces a lack or an increase of detection by SNA I and RCA_{120}, respectively. The removal of terminal NeuAc followed by lectin detection is illustrated by the on blot glycan analysis of human alpha 1 antichymotrypsin (**Fig. 3**).

Terminal β1,4-galactose enzymatic hydrolysis *(7)*

The enzyme used for degalactosylation is a β-galactosidase from *Aspergillus oryzae* and is specific for terminal β1,4- and β1,6-galactoses *(8)* (*see* **Note 15**). *A. oryzae* β-galactosidase needs to be purified from the commercial preparation *(8)*, prior to its use for *N*-glycan degalactosylation.

1. Purify *A. oryzae* β-galactosidase by applying it to an ion-exchange chromatography on S Sepharose Fast-flow column equilibrated in 10m*M* sodium acetate, pH 4.6. Elute β-galactosidase using a linear gradient of 0–0.5 *M* sodium chloride. Check the purity of the enzyme by gel filtration on Sephacryl S200 HR equilibrated in 50 m*M* Tris-HCl, pH 7.5, complemented with 0.1 *M* sodium chloride, or by SDS-PAGE. Evaluate the activity of the enzyme preparation by incubating a fraction of the purified *A. orizae* with 5 m*M* pNP-β-Gal in 50 mM sodium acetate, pH 5.0. One milliunit is defined as the amount of β-galactosidase inducing the liberation of 1 nmol of 4-nitrophenol per minute *(9)*.
2. Dissolve 10 mg of glycoprotein (*see* **Note 16**) in 0.5 mL of 50 m*M* sodium acetate, pH 5.0, and incubate overnight with 4.2 U of purified β-galactosidase from *A. orizae*.

Table 2
Exoglycosidases and glycosyltranferases used for *N*-glycan sequencing prior to on blot detection

Enzyme	Action	Occurrence of the substrate	Detection	Information
Neuraminidase	Removal of NeuAc from: NeuAc-α2,3-GlcNAc- NeuAc-α2,6-GlcNAc-	Complex N-glycans in mammals	- Loss of reactivity with MAL II ou SNA. - Increased reactivity with RCA_{120}	Presence of terminal NeuAc
Galactosidase	Removal of Gal from: Gal-β1,4-GlcNAc-	Complex N-glycans in mammals Complex N-glycans in plants expressing a g alactosyltransferase	- Loss of reactivity with RCA_{120}	Presence of terminal Gal
Galactosyl transferase	Addition of a β1,4-Gal on a terminal β1,2-GlcNAc	Complex N-glycans in mammals and plants	- Gain of reactivity with RCA_{120}	Presence of terminal GlcNAc

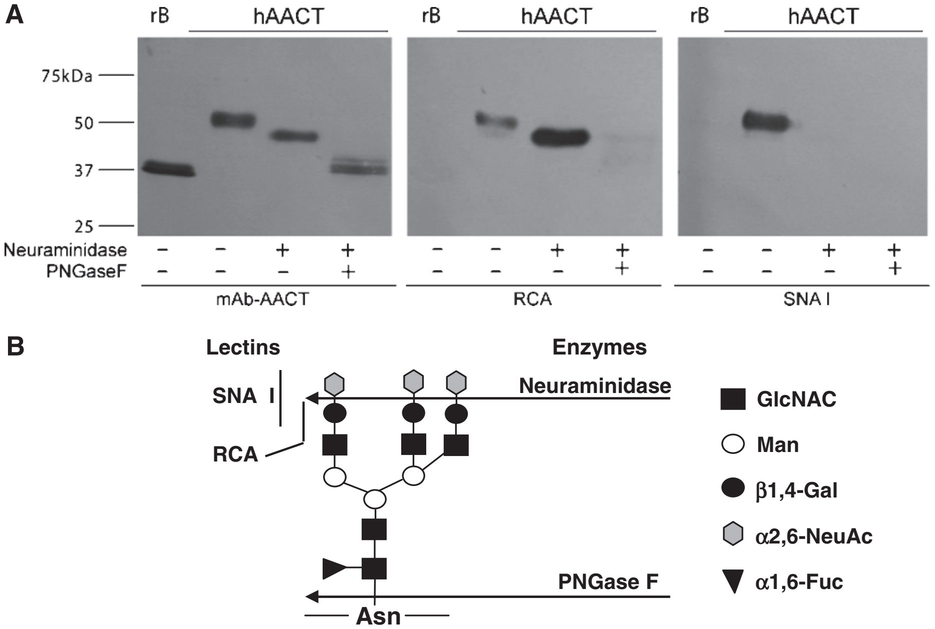

Fig. 3. Human α1 anti-trypsin (hAACT) is higly glycosylated with complex *N*-glycans containing sialic acids. (A) Human AACT (hAACT) was successively digested with neuraminidase and peptide *N*-glycosidase F (PNGase F). All samples were analysed by immuno- and affinodetection with AACT specific antibodies (mAb-AACT), *Ricinus communis* agglutinin (RCA) and *Sambucus nigra* lectin I (SNA I). Bacterial recombinant AACT (rb) was used as a non-glycosylated control. (B) Suggested *N*-glycan structure of AACT as supported by the exoglycosidase digestion combined to lectin binding assays.

3. Dilute then the sample in a suitable SDS-PAGE buffer prior to electrophoretic migration, Western blotting and detection using RCA_{120} (*see* **Subheading 3.2.1.1**). A correct degalactosylation induces a loss in RCA_{120} detection.

3.2.3.2. IN VITRO MODIFICATION WITH GLYCOSYLTRANSFERASES

Glycosyltranferases can also be used for the characterization of *N*-linked glycans. This is illustrated through the following exemple.

To specifically detect terminal GlcNAc, a Gal residue can be added to the non-reducing end of *N*-glycans by a β1,4-galactosyltransferase from bovine milk (*see* **Note 17**). The resulting *N*-linked glycan is detectable by RCA_{120} (*see* **Table 2**).

1. Dissolve 50 µg of glycoprotein (*see* **Note 18**) in 1 mL of 100 m*M* sodium cacodylate, pH 6.4, containing 5 m*M* UDP-galactose and 5 mM $MnCl_2$ (*see* **Note 19**). Incubate for 24h at 37°C.
2. Dilute then the sample in a suitable SDS-PAGE buffer prior to electrophoretic migration, Western blotting and detection using RCA_{120} (*see* **Subheading 3.2.1.1**). β1,4-Galactosylation of terminal GlcNAc residues renders the glycoprotein detectable by RCA_{120}.

4. Notes

1. Do not freeze Endo H as this inactivates the protein.
2. The experimenter should be careful in the blot interpretation, as WGA recognizes GlcNAc in *N*-linked glycans, as well as *O*-linked GlcNAc.
3. The solution used for blocking binding sites on the nitrocellulose needs to be devoid of glycoproteins. This is why we recommend using Tween-20 to coat the nitrocellulose ***(10)*** in this procedure.
4. Controls for specificity:
   a. It might be advisable to blot the proteins cited in **Table 1** on the membrane ("Suggested positive controls"), to get positive controls for the affinodetection.
   b. Lectin binding specificity must also be checked by running affinodetection in the presence of 0.3 *M* inhibitory sugar (**Table 1**).
5. Core α1,3-fucosylation has been shown to be reponsible for cross-reactions between *Drosophila melanogaster* neuronal proteins and horseradish peroxidase. Anti-horseradish peroxidase antibodies are used as markers for neurons of *D. melanogaster* ***(11)***.
6. No immunsera or antibodies are commercially available to detect mammalian *N*-glycans.
7. Control for *N*-glycan specificity of the immunodetection: it might be wise to check for the specificity of sera toward *N*-glycans attached to the protein studied. This can be done by performing a mild periodate oxidation on the blot prior to immunodetection. Mild periodate treatment oxidizes glycans and abolishes any recognition of the glycoprotein by the anti-glycan antibodies. Any remaining signal will be the consequence of a protein backbone antibody/recognition ***(12)***.
   a. After saturation with gelatin, incubate the blot in a 100 m*M* sodium acetate buffer, pH 4.5, containing 100 m*M* sodium metaperiodate for 1 h in the dark at room temperature, changing the incubation solution after 30 min.
   b. Incubate the blot in PBS containing 50 m*M* sodium borohydride for 30 min at room temperature.
   c. Rinse the blot with TBS, saturate for 15 min with TBS containing 1% gelatin, and perform the immunodetection, as described in **Subheading 3.2.1.1**.
8. Controls for fucose or xylose specificity: some proteins can be used as positive controls for the *N*-glycan immunodetection. Phospholipase A2 from honeybee venom contains the α1,3-fucose residue, and is devoid of β1,2-xylose ***(13)***. PHA-L is a glycoprotein containing both β1,2-xylose and α1,3-fucose ***(14)***.

9. Some authors have shown some success at deglycosylating an entire glycoprotein. The result of this experiment is glycoprotein-dependent and the researcher should be aware that most glycoproteins will not be deglycosylated by PNGase A, unless previously digested by a protease.
10. To overcome the problem caused by the presence of α1,3-fucose on a plant glycoprotein, the researcher can express it, if its cDNA is available, in a plant system devoid of complex *N*-linked glycans. Such a system can be the *cgl* mutant, an *Arabidopsis thaliana* mutant in which *N*-acetylglucosaminyltransferase 1, the enzyme responsible for the addition of the 1st terminal GlcNAc, has been impaired. This mutant accumulates secreted glycoproteins presenting *N*-glycans matured only to the Man5 form ***(15)***. Researchers can also express the glycoprotein of interest in *A. thaliana* or in the moss *Physcomytrella patens*, in which the genes encoding xylosyltransferase and fucosyltranferase, the two enzymes responsible for the addition of β1,2-xylose and α1,3-fucose, have been knocked-out (***16***; ***17***).
11. The role of Nonidet P40 is to complex free SDS.
12. Although several xylosidases and fucosidases are proposed by suppliers, they have been used only rarely in the literature, and never in our laboratory. Therefore, they will not be detailed here.
13. It is also possible to chemically eliminate the terminal NeuAc by incubationg the glycoprotein in 0.1 N HCl at 80°C for 1h.
14. The experiment has been settled on human alpha 1 antichymotrypsine (***29***), a glycoprotein bearing 4 *N*-linked glycans ***(18)***.
15. As β1,6-galactose occurs rarely in *N*-glycans, one can consider the β-galactosidase from *A. oryzae* specific for terminal β1,4-galactose residues.
16. The protocole has been established using human transferrin ***(7)***, a glycoprotein bearing 2 *N*-linked glycans ***(19)***.
17. β1,4-galactosyltranferase from milk adds a Gal preferentially on the β1,2-GlcNAc linked to the α1,3-mannose of the *N*-glycan core ***(20)***.
18. The protocole has been established using a mouse recombinant antibody expressed in plants ***(21)***.
19. The experiment presented here is a "cold" technique. The same method can be reproduced using radioactive UDP-Gal. The addition of galactose will visualized by autoradiography.
20. Free GlcNAc is a ligand of WGA and will displace the bound glycoproteins from the immobilized lectin.

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A Lectin-Binding Assay for the Rapid Characterization of the Glycosylation of Purified Glycoproteins

Mohammad T. Goodarzi, Angeliki Fotinopoulou, and Graham A. Turner

1. Introduction

Most proteins have carbohydrate chains (glycosylation) attached covalently to various sites on their polypeptide backbone. These posttranslational modifications, which are carried out by cytoplasmic enzymes, confer subtle changes in the structure and behavior of a molecule, and their composition is very sensitive to many environmental influences ***(1–3)***. There is increasing interest in determining the glycosylation of a molecule because of the importance of glycosylation in affecting its reactivity ***(1, 4, 5)***. This is particularly true in the production of therapeutic glycoproteins by recombinant methods, in which glycosylation can be determined by the type of host cell used or the production process employed ***(2)***. Glycosylation is also important in disease situations in which changes in the carbohydrate structure can be involved in the pathological processes ***(3,6,7,8)***. Unfortunately, the glycosylation of proteins is very complex; there are variations in the site of glycosylation, the type of amino acid–carbohydrate bond, the composition of the chains, and the particular carbohydrate sequences and linkages in each chain ***(3, 4)***. In addition, within any population of molecules there is considerable heterogeneity in the carbohydrate structures (glycoforms) that are synthesized at any one time ***(1)***. This is typified by some molecules showing increased branching, reduced chain length, and further addition of single carbohydrate moieties to the internal chain.

To unravel completely the complexities of the glycosylation of a molecule is a substantial task, which requires considerable effort and resources. However, in many situations this is unnecessary, because only a limited amount of information,

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on a single or a group of structural features, is needed. Lectins can be useful for this purpose. These substances are carbohydrate binding proteins with particular specificity ***(9)***. Although their specificity is not absolute, there is usually one carbohydrate or group of carbohydrates to which the lectin binds with a higher affinity than the rest of the group. The major carbohydrate specificity of a number of commonly used lectins is shown in **Table 1**.

Lectins have previously been used for investigating glycoprotein glycosylation by incorporating them into existing technologies such as affinity chromatography, blotting, and electrophoresis. Although these modifications give workable methods, they frequently use large amounts of lectin, which is expensive, requires considerable technical skill, cannot handle large numbers of specimens, and provides only semiquantitative results. For a more detailed discussion of these methods the reader should refer to a previous review ***(3)***.

Another approach is to use lectins in the familiar sandwich enzyme-linked immunosorbent assay (ELISA) technology in multiwell plates. Procedures of this type have been described for the measurement of particular carbohydrate structures in α-fetoprotein ***(10)***, glycodelin ***(11)***, haptoglobin (HP) ***(12)***, human chorionic gonadotrophin (hCG) ***(13)***, α1-acid glycoprotein (AGP) ***(14)***, fibronectin ***(15)***, c-*erb*-B2 ***(16)***, immunoglobulins (IgG) ***(17)***, mucins ***(18)***, plasminogen (Pg) activator ***(19)***, and transferrin (TF) ***(20)***. In this method, the lectin is used either to capture the molecule of interest or to identify it. An antibody is used as the other partner in the sandwich and the degree of binding is measured by the presence of an enzyme label on the identifier. Both configurations suffer from the disadvantage that the lectin may bind to carbohydrate determinants on the immunoglobulin used as the antibody. Furthermore, using the lectin as the capture molecule, the immobilized lectin may bind to glycans of other glycoproteins

Table 1
Specificity of Different Lectins

Lectin	Abbreviation	Specificity
Concanavalin A	ConA	Mannose α1–3 or mannnose α1–6
Datura stramonium agglutinin	DSA	Galactose β1–4 *N*-acetylglucosamine
Aleuria aurantia agglutinin	AAA	Fucose α1–6 *N*-acetylglucosamine
Lens culinaris agglutinin	LCA	Mannose α1–3 or mannose α1–6 (Fucose α1–6 GlcNAc is also required)
Lotus tetragonolobus agglutinin	LTA	Fucose α1–2 galactose β1–4 [Fucose α1–3] *N*-acetylglucosamine
Sambucus nigra agglutinin	SNA	*N*-acetylneuraminic acid α2–6 galactose
Maackia amurensis agglutinin	MAA	*N*-acetylneuraminic acid α2–3 galactose
Peanut agglutinin	PNA	Galactose β1–3 *N*-acetylgalactosamine on O-linked chains

in the sample and these will compete with the molecule of interest for the available binding sites. When immobilized antibody is used as the capture molecule, care must be taken to ensure that the antibody is not binding to the same determinant as that reacting with lectin.

The lectin binding assay (LBA) described herein *(21)* overcomes many of the disadvantages with the previous lectin immunoassays. It was developed from a previously reported procedure *(22)*. A purified glycoprotein is absorbed onto the plastic surface of a well in a microtiter plate. After the unbound protein is removed by washing, uncoated sites on the plate are blocked using a non-ionic detergent. A lectin labeled with digoxigenin (DIG) or biotin is added and allowed to interact with the carbohydrate on the absorbed glycoprotein. Unbound lectin is removed by further washing and the amount of bound lectin is measured by adding an anti-DIG antibody or streptavidin conjugated to an enzyme. Streptavidin has a very high affinity for biotin. Following further washing, the bound enzyme is used to develop a color reaction by the addition of the appropriate substrates. The principle of the procedure is summarized schematically in **Fig. 1**.

Using this method it is possible to rapidly screen multiple specimens, with high sensitivity and excellent precision. In addition, very small amounts of

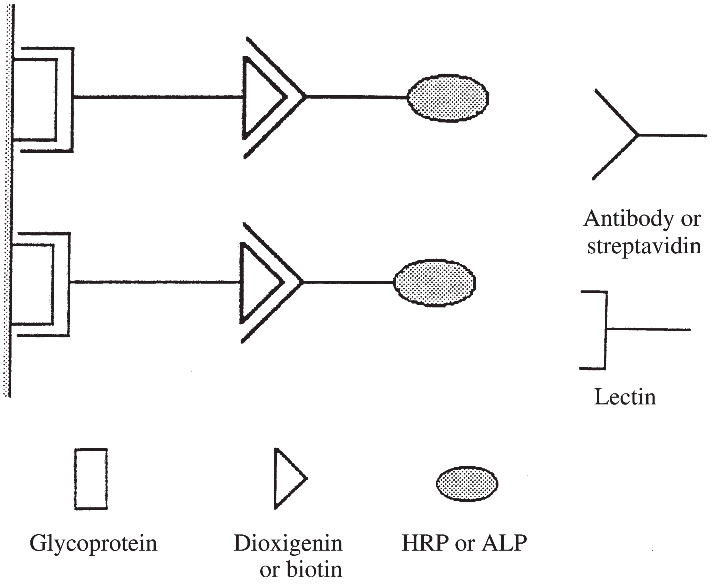

Fig. 1. Schematic diagram of the interactions in the LBA.

lectin are used, background absorbances are low, and the procedure does not require a high degree of technical skill other than some experience with micropipets and ELISA. Because such small amounts of glycoprotein are needed, a glycoprotein can be rapidly purified by a batch affinity chromatography method ***(23, 24)***. The LBA has successfully been applied to the investigation of the glycosylation of purified alpha-1-proteinase inhibitor (API) ***(24)*** and HP ***(25)*** using concanavalin A (ConA), *Maackia amurensis* agglutinin (MAA), and *Sambucus nigra* agglutinin (SNA). Other studies of API and HP have been carried out using *Lens culinaris* agglutinin (LCA) and *Lotus tetragonolobus* agglutinin (LTA) (unpublished observations). If a panel of lectins is used, an overall picture of the carbohydrate structure of a glycoprotein can be built up very quickly and cheaply. Furthermore, subtle differences in the glycosylation of the same glycoprotein in different situations can be identified, for example, different diseases ***(24, 25)*** or between different batches of a recombinant protein ***(26)***.

2. Materials

2.1. Reagents

1. Tris-buffered saline (TBS): 125 m*M* Tris-HCl, pH 7.5, containing 100 m*M* NaCl pH 7.5.
2. Control glycoprotein (*see* **Notes 1** and **2**).
3. Sample glycoprotein (*see* **Notes 2** and **3**).
4. Tween–TBS (TTBS): 125 m*M* Tris-HCl, pH 7.5, containing 100 m*M* NaCl and 0.1% (v/v) Tween 20.
5. Tris cations (TC): 1 m*M* Tris-HCl, pH 7.5, containing 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, and 0.1% (v/v) Tween 20.
6. DIG-labeled lectins (Roche Molecular Biochemicals): Lectin-DIG dissolved in TC (*see* **Note 3**).
7. Biotin-labeled lectins (Sigma): LCA-biotin and LTA-biotin dissolved in TC (*see* **Note 3**).
8. Anti-DIG antibody conjugated with horseradish peroxidase (HRP) (Roche Molecular Biochemicals): 50 mU/mL (Fab fragment) in TTBS (*see* **Note 4**).
9. Streptavidin-alkaline phosphatase (SALP) (Sigma): 1 ng/µL (1.3 mU/µL) in TTBS.
10. Citrate buffer: 34.8 m*M* citric acid, 67.4 m*M* Na_2HPO_4, pH 5.0.
11. Diethanolamine buffer: 100 m*M* diethanolamine, pH 9.8, containing 1 m*M* $MgCl_2$. Store in the dark at 4°C and prepare monthly.
12. *O*-Phenylenediamine (OPD): 37 m*M* solution which is stored in 1 mL aliquots at −20°C.
13. *p*-nitrophenylphosphate (PNPP) (BDH, Atherstone, UK).
14. Hydrogen peroxide: 3% (v/v) solution prepared by diluting concentrated (30%) H_2O_2 1:10 with deionized water. Prepared monthly and stored at 4°C.

15. Color reagent:
    a. HRP: 9 mL citrate buffer + 1 mL of OPD + 50 mL of 10% H_2O_2. Make up fresh 10 min before use. H_2O_2 is added just prior to use.
    b. SALP: 20 mL of diethanolamine buffer + 20 mg of PNPP. Prepare fresh.
16. 1.25 *M* Sulfuric acid (H_2SO_4).
17. 1 *M* Sodium hydroxide (NaOH).

All other reagents were of analytical grade or better (BDH or Sigma), and prepared as required. All solutions were prepared with double-distilled deionized water (Millipore, Bedford, MA).

2.2. Equipment

1. Multiwell plastic plate: 96-Well plate (Immunolon 4, Dynex, Chantilly, VA).
2. Plate reader: Multiskan MCC/340 (Titerteck [McLean, VA]).
3. Multichannel micropipet: Finnpipet, Labsystem, Finland.

3. Methods

1. For each lectin add 100 µL of sample glycoprotein in triplicate to a multiwell plate, and for each batch of samples, add 100 µL of control glycoprotein or TBS in triplicate.
2. Incubate the plate for 2 h at 37°C. Pour off liquid by turning upside down, and tap on a pad of tissue paper to dry completely.
3. Add 200 µL of TTBS to each well, pour off liquid, and dry again. Repeat this operation 3×.
4. Add 200 µL of TTBS to each well and incubate for 1 h at 37°C and then overnight at 4°C.
5. Wash each well 3× with 200 mL of TC, removing liquid as described in **step 2**.
6. Add 200 mL of DIG-lectin or biotin-lectin to each well and incubate for 1 h at 37°C.
7. Wash each well 4× with 200 mL TTBS, removing liquid as described in **step 2**.
8. Add 150 mL anti-DIG/HRP or SALP to each well and incubate the DIG/HRP for 1 h at room temperature and the SALP for 1 h at 37°C.
9. Wash each well 4× with TTBS, removing liquid as described in **step 2**.
10. Add 100 µL of color reagent to each well as appropriate.
11. Leave plate for 10–30 min (*see* **Note 5**) at room temperature (in the dark for HRP).
12. Stop reaction:
    a. For HRP, add 100 µL of H_2SO_4 to each well.
    b. For SALP, add 100 µL of NaOH to each well.
13. Read the absorbance for each well with plate reader:
    a. For HRP, 492 nm.
    b. For SALP, 405 nm.
14. Calculate the mean of triplicate measurements and subtract the absorbance value (OD) of the blank from the values of all samples and controls.

4. Notes

1. A pool of purified glycoprotein that reacts with the lectin is recommended as a positive control in the assay and also for preliminary experiments to develop assay conditions. Various serum glycoproteins can be obtained commercially in a purified form for this purpose, but with other glycoproteins this may not be possible, and suitable material may have to be prepared in the laboratory.
2. Sample and control glycoproteins can be conveniently purified for assay by extracting with an antibody coupled to Sepharose. We have described a batch method that is very rapid, can handle many specimens, produces very pure protein, and works for a number of different glycoproteins ***(23,24)***. Although the yield from this procedure can be low (5–20%), this does not matter because only low amounts of glycoprotein are needed in the assay. A similar method could be equally well adapted for the extraction of other types of glycoprotein.
3. For each combination of lectin and protein, preliminary experiments are carried out to determine the optimum concentration of protein required to coat the well. This is done by coating the well with different amounts of protein (1–1000 ng) and probing with a lectin solution of 1 μg/mL ***(22)***. The protein concentration is chosen that gives an OD value of approx 1. If this value cannot be established, the lectin concentration is increased (e.g., 1.5 μg/mL) or decreased (e.g., 0.5 μg/mL) as required, and the protein is reassayed. **Table 2** shows the optimum glycoprotein concentrations for various lectins we have previously studied. The concentrations of Con A, LCA, LTA, MAA, and SNA used in these experiments were 1, 0.5, 1, 1.5, and 1 μg/mL, respectively.
4. The activity of anti-DIG HRP varies from batch to batch and should be routinely checked. This reagent is usually used at a dilution of 1:3000 to 1:6000.

Table 2
Optimum Protein Coating Concentration for Different Lectins and Different Serum Glycoproteins

	Optimum protein concentration, μg/mL			
Lectin	API	HP	TF	AGP
ConA	0.15	0.05	0.15	2.50
SNA	0.15	0.05	0.07	0.50
MAA	1.00	0.50	3.00	0.50
LCA	1.50	0.50	NR	ND
LTA	1.50	1.00	NR	ND
DSA	0.05	0.01	0.35	0.06

NR, no reactivity; ND, not determined.

5. The time required to develop the color reaction depends on the reactivity of lectin with the particular protein being investigated. For some lectins (e.g., Con A and SNA) it is between 10 and 30 min whereas for other lectins (e.g., LTA, MAA) it is as long as 120 min.
6. To minimize background, TBS and TTBS must be filtered through a 0.2-µm membrane (Tuffryn membrane). The absorbances (without glycoprotein but with all other reagents) for Con A, SNA, and MAA were (mean ± SD, no. of observations) 0.28 ± 0.02, 9, 0.16 ± 0.03, 9; 0.14 ± 0.02,9 respectively.
7. The method gives very good reproducibility. For example, in the case of API, the interassay precision using ConA, SNA, and MAA was 2.2%, 4.5%, and 6.4%, respectively, and the intraassay precision for this glycoprotein with the same lectins was 1.5, 3 and 5%, respectively.
8. The specificity of each lectin can be checked by assaying the glycoprotein in the presence of a competitive sugar. For example, in the presence of 100 m*M* α-D-methyl mannoside, a competitive inhibitor for ConA, the absorbance obtained for API is reduced from 2 to 0.09. Alternatively, glycoproteins can be used that are known to lack the carbohydrate grouping under investigation, for example, carboxypeptidase Y-MAA and TF-LTA.
9. The method is only semiquantitative, and if high absorbance values are obtained for a glycoprotein this suggests that it has high amounts of a particular carbohydrate grouping, and vice versa. Positive and negative controls must always be run in the assay, because it does not use standards. The method is at its most useful when comparing different samples in the same assay. This is illustrated in **Table 3**, which shows the lectin binding characteristics for Hp from healthy individuals and cancer patients. The results are interpreted according to the known properties of each lectin *(27)*. Therefore, in cancer, the branching of Hp is increased, there is more α2–3 *N*-acetyneuraminic acid (Neu5A), and the α2–6 Neu5A content is unchanged. On the other hand, with API in cancer, the branching decreases, the α2–6 Neu5A con-

Table 3
Analysis of Purified API and Hp in the LBA using ConA, MAA, and SNA

		Lectin[b] ($OD_{492\ nm}$)		
Glycoprotein	Source	ConA	SNA	MAA
Hp	Healthy	1.40 ± 0.35	1.70 ± 0.22	0.18 ± 0.07
	Cancer[a]	0.86 ± 0.15	1.69 ± 0.29	0.45 ± 0.10
API	Healthy	1.29 ± 0.28	1.31 ± 0.19	0.47 ± 0.08
	Cancer[a]	1.87 ± 0.14	1.95 ± 0.09	0.23 ± 0.07

[a] Cancer specimen from patients with stage III/IV ovarian carcinoma.

[b] Mean ± SD calculated from the values of 8 healthy individuals or 12 cancer patients.

Table 4
Effect of Neuraminidase Treatment of Immobilized Glycoproteins on Their Lectin Binding Activity

	Lectin binding ($OD_{492\ nm}$)							
	Before NANase				After NANase			
Glycoprotein	ConA	MAA	PNA	SNA	ConA	MAA	PNA	SNA
Fetuin	0.81	1.15	0.04	2.78	1.08	0.09	1.23	0.08
IgG	1.61	0.06	—	1.62	1.29	—	0.11	0.34
Pg	1.45	1.37	0.53	1.13	0.99	0.32	1.24	0.25

Immobilized proteins were treated with 9 mU of neuraminidase (*Vibrio cholerae*, Roche Molecular Biochemicals) for 16 h at 37°C. After treatment the well was washed 4× with TC prior to measurement of lectin binding. Fetuin and IgG are from Sigma and Pg is from Biopool, Sweden.

tent increases, and the α2–3 Neu5A content decreases. All these changes are consistent with the reported monosaccharide composition of these specimens ***(23, 24)***, and the known carbohydrate structures present in *N*-glycans ***(4)***. It is important to emphasize that the method does not give an indication of the glycosylation of individual glycoforms in a population, but represents the overall glycosylation.

10. More information about carbohydrate structure can be obtained from the LBA by treating the immobilized glycoprotein with a glycosidase, for example, neuraminidase (NANase), galactosidase. **Table 4** shows the effect of NANase treatment (the glycosidase that removes terminal Neu5Ac from oligosaccharide chains] on the binding of ConA, MAA, PNA, and SNA to IgG, fetuin, and plasminogen (Pg). The presence of terminal α2–3 and α2–6 Neu5Ac can be clearly shown using lectins and NANase treatment. Furthermore, the presence of a cryptic grouping (galactose β1–3 *N*-acetylgalactosamine) on *O*-Linked chains can be demonstrated on fetuin and Pg with PNA after the removal of Neu5Ac. *O*-Linked chains were not detected on IgG, which appears also to lack α2–3 Neu5Ac.
11. Because OPD is carcinogenic, the noncarcinogenic substance, 3,3′,5,5′-tetramethyl benzidine (dihydrochloride) can be used as a substrate for HRP ***(28)***.

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Chemical Methods of Analysis of Glycoproteins

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

The first analysis of glycoconjugates that often needs to be carried out is to see if they indeed contain sugar. For glycoproteins in gels or oligosaccharides in solution, this can be readily achieved by periodate oxidation at two concentrations, the first to detect sialic acids, and the second, any monosaccharide that has two free vicinal hydroxyl groups ***(1)***. Periodate cleaves between the hydroxyl groups to yield reactive aldehydes, which can be detected by reduction with NaB^3H_4 or coupled to high sensitivity probes available in commercial kits, e.g., from Boeringher Mannheim (Mannheim, Germany) or Oxford GlycoSciences (Abingdon, UK). In solution, a quick spot assay can be carried out for the presence of any monosaccharide or oligosaccharide having a C-2 hydroxyl group by visualization with charring by phenol/sulfuric acid reagent ***(2)***. These methods are relatively specific for mono/oligosaccharides ***(3)***.

2. Materials

2.1. Periodate Oxidation

1. 0.1 *M* Acetate buffer, pH 5.5, containing 8 or 15 m*M* sodium periodate.
2. Ethylene glycol.
3. 0.1 *M* Sodium hydroxide.
4. Reducing agent: sodium borohydride, tritiated sodium borohydride, or sodium borodeuteride.
5. Glacial acetic acid.
6. Methanol.

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2.2. Phenol Sulfuric Acid Assay

1. H_2O (HPLC-grade).
2. 4% Aqueous phenol.
3. Concentrated H_2SO_4.
4. 1 mg/mL galactose.
5. 1 mg/mL mannose.

3. Methods

3.1. Periodate Oxidation

1. Dissolve 0.1–1.0 mg glycoprotein in 2 mL of acetate buffer containing sodium periodate (15 m*M* for all monosaccharides, 8 m*M* for alditols, and 1 m*M* specifically for oxidation of sialic acids).
2. Carry out the periodate oxidation in the dark at room temperature for 1 h for oligosaccharides, or at 4°C for 48 h for alditols, or 0°C for 1 h for sialic acids (*see* **Note 1**).
3. Decompose excess periodate by the addition of 25 µL of ethylene glycol, and leave the sample at 4°C overnight.
4. Add 0.1 *M* sodium hydroxide (about 1.5 mL) until pH 7.0 is reached.
5. Reduce the oxidized compound with 25 mg of $NaB[^3H]_4$ at 4°C overnight (*see* **Note 2**).
6. Add acetic acid to pH 4.0, and concentrate the sample to dryness.
7. Remove boric acid by evaporations with 3 × 100 µL methanol (*see* **Note 3**).

3.2. Phenol/Sulfuric Acid Assay

1. Aliquot a solution of the unknown sample containing a range of approx 1 µg/10 µL into a microtiter plate along with a range of concentrations of a hexose standard (galactose or mannose, usually 1–10 µg).
2. Add 25 µL of 4% aquenous phenol to each well, mix thoroughly, and leave for 5 min (*see* **Note 4**).
3. Add 200 µL of H_2SO_4 to each well, and mix prior to reading on a microtiter plate reader at 492 nm (*see* **Note 5**).

4. Notes

1. It is important that the periodate oxidation is carried out in the dark to avoid unspecific oxidation. The periodate reagent has to be prepared fresh, since it is degraded when exposed to light.
2. The reactive aldehydes can also be detected by coupling to an amine-containing compound, such as digoxigenin ***(1)***.
3. Addition of methanol in an acidic environment leads to the formation of volatile methyl borate.
4. Do not overfill wells during the hexose assay, since the conc. H_2SO_4 will severely damage the microtiter plate reader if spilled.

5. Exercise care when adding the conc. H_2SO_4 to the phenol/alditol mature, since it is likely to "spit," particularly in the presence of salt.

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Monosaccharide Analysis by HPAEC

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1. Introduction

Once the presence of monosaccharides has been established by chemical methods (*see* Chapter 128), the next stage of any glycoconjugate or polysaccharide analysis is to find out the amount of sugar and monosaccharide composition. The latter can give an idea as to the type of oligosaccharides present and, hence, indicate further strategies for analysis ***(1)***. Analysis by high-pH anion-exchange chromatography (HPAEC) with pulsed electrochemical detection, that is described here, is the most sensitive and easiest technique ***(2)***. If the laboratory does not have a biocompatible HPLC available that will withstand high salt concentrations, a sensitive labeling technique can be used with gel electrophoresis ***(3)***, e.g., that marketed by Glyko Inc. (Navato, CA) or Oxford GlycoSciences (Abingdon, UK), to include release of oligosaccharides/monosaccharides and labeling via reductive amination with a fluorescence label ***(4)***.

2. Materials

1. Dionex DX500 (Dionex Camberley, Surrey UK) or other salt/biocompatible gradient HPLC system (titanium or PEEK lined), e.g., 2 Gilson 302 pumps with 10-mL titanium pump heads, 802Ti manometric module, 811B titanium dynamic mixer, Rheodyne 7125 titanium injection valve with Tefzel rotor seal (Gilson Medical Electronics, Villiersle-Bel, France).
2. CarboPac PA1 separator (4 × 250 mm) and PA1 guard column (Dionex).
3. Pulsed amperometric detector with Au working electrode (Dionex), set up with the following parameters:
    a. Time = 0 s E = + 0.1 V
    b. Time = 0.5 s E = + 0.1 V
    c. Time = 0.51 s E = + 0.6 V

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   d. Time = 0.61 s E = + 0.6 V
   e. Time = 0.62 s E = − 0.8 V
   f. Time = 0.72 s E = − 0.8 V
4. Reagent reservoir and postcolumn pneumatic controller (Dionex).
5. High-purity helium.
6. 12.5 *M* NaOH (BDH, Poole, UK).
7. Reagent grade sodium acetate (Aldrich, Poole, UK).
8. HPLC-grade H_2O.
9. 2 *M* Trifluoroacetic acid HPLC-grade.
10. 2 *M* HCl.
11. Dowex 50 W × 12 H^+ of cation-exchange resin.
12. 3.5-mL screw-cap septum vials (Pierce, Chester, UK) cleaned with chromic acid (2 L H_2SO_4/350 mL H_2O/100 g Cr_2O_3) (**Use care! extremely corrosive,** *see* **Note 1**), and coated with Repelcote (BDH, Poole, UK).
13. Teflon-backed silicone septa for 3.5-mL vials (Aldrich).

3. Method

1. Dry down the glycoprotein (10 µg) or oligosaccharide (1 µg) in a clean screw-top vial with Teflon-backed silicone lid insert (*see* **Note 2**).
2. Hydrolyze in an inert N_2 atmosphere for 4 h at 100°C with 2 *M* HCl.
3. Dry the hydrolyzate and re-evaporate three times with HPLC-grade H_2O.
4. Purify on a 1-mL Dowex 50 W × 12 H^+ column eluted in water.
5. Dry down the monosaccharides ready for injection onto the HPLC system.
6. Prepare the following eluants:
   Eluant A = 500 mL HPLC-grade H_2O.
   Eluant B = 500 mL of 50 m*M* NaOH, 1.5 m*M* sodium acetate.
   Eluant C = 100 mL of 100 m*M* NaOH.
7. Degas the eluants by bubbling through helium.
8. Place the postcolumn reagent in a pressurized reagent reservoir (300 mm NaOH) and use the pneumatic controller to adjust helium pressure to give a flowrate of 1 mL/min (approx 10 psi).
9. Equilibrate the column with 98% eluant A and 2% eluant B at a flowrate of 1 mL/min.
10. Add the postcolumn reagent between column and detector cell at a flowrate of 1 mL/min via a mixing tee.
11. Inject approx 100 pmol of monosaccharide and elute isocratically as follows: eluant A = 98%; eluant B = 2%; flowrate = 1 mL/min; for 30 min.
12. Calculate monosaccharide amounts by comparison with a range of known monosaccharide standards run on the same day with deoxyglucose as an internal standard. From this, it is possible to infer the type and amount of glycosylation of the glycoprotein.
13. Regenerate the column in eluant C for 10 min at 1 mL/min (*see* **Note 3**).
14. Re-equilibrate the column with 98%A/2%B before the next injection.

15. At the end of the analysis, regenerate the column in eluant C, and flush pumps with H_2O (*see* **Note 4**).

4. Notes

1. If required, an equivalent detergent-based cleaner may be used.
2. Use polypropylene reagent vessels as far as possible for HPAEC-PAD because of the corrosive nature of the NaOH, and to minimize leaching of contaminants from the reservoirs.
3. Some drift in retention times may be observed during the monosaccharide analysis. This can be minimized by thorough regeneration of the column and use of a column jacket to maintain a stable column temperature.
4. Failure to wash out the eluants from the pumps at the end of an analysis may result in crystallization and serious damage to the pump heads.

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Monosaccharide Analysis by Gas Chromatography (GC)

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

Although the methods given in Chapter 129 can give an approximate idea of oligosaccharide amount or composition, they would not be able to distinguish the multiple monosaccharides and substituents present in nature. For this, the high resolution of gas chromatography (GC) is required ***(1,2)***. The most unambiguous results are provided by analysis of trimethylsilyl ethers (TMS) of methyl glycosides with on line mass selective detection (MS).

2. Materials

1. 0.5 *M* Methanolic HCl (Supelco, Bellefont, PA).
2. Screw-top PTFE septum vials.
3. Phosphorus pentoxide.
4. Silver carbonate.
5. Acetic anhydride.
6. Trimethylsilylating (TMS) reagent (Sylon HTP kit, Supelco: pyridine hexamethyldisilazane, trimethylchlorosilane; **use care; corrosive**).
7. Toluene stored over 3-Å molecular sieve.
8. GC apparatus fitted with flame ionization or MS detector and column, e.g., for TMS ethers 25 m × 0.22 mm id BP10 (SGE, Austin, TX) 30 m × 0.2 mm id ultra-2 (Hewlett Packard, Bracknell, Berkshire, UK).

3. Method

1. Concentrate glycoproteins or oligosaccharides containing 1–50 μg carbohydrate and 10 μg internal standard (e.g., inositol or perseitol) in screw-top septum vials. Dry under vacuum in a desiccator over phosphorus pentoxide.

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2. Place the sample under a gentle stream of nitrogen, and add 200 μL methanolic HCl (*see* **Note 1**).
3. Cap immediately, and heat at 80°C for 18 h (*see* **Note 2**).
4. Cool the vial, open, and add approx 50 mg Silver carbonate.
5. Mix the contents, and test for neutrality (*see* **Note 3**).
6. Add 50 μL acetic anhydride, and stand at room temperature for 4 h in the dark (*see* **Note 4**).
7. Spin down the solid residue (*see* **Note 5**), and remove the supernatant to a clean vial.
8. Add 100 μL methanol, and repeat **step 7**, adding the supernatants together.
9. Repeat **step 8** and evaporate the combined supernatants under a stream of nitrogen.
10. Dry over phosphorus pentoxide before adding 20 μL TMS reagent.
11. Heat at 60°C for 5 min, evaporate remaining solvent under a stream of nitrogen, and add 20 μL dry toluene.
12. Inject onto a capillary GC column with 14 psi He head pressure and a temperature program from 130 to 230°C over 20 min and held at 230°C for 20 min.
13. Calculate the total peak area of each monosaccharide by adding individual peaks and dividing by the peak area ratio of the internal standard. Compare to standard curves for molar calculation determination.

4. Notes

1. The use of methanolic HCl for cleavage of glycosidic bonds and oligosaccharide-peptide cleavage yields methyl glycosides and carboxyl group methyl esters, which gives acid stability to the released monosaccharides, and thus, monosaccharides of different chemical lability can be measured in one run. If required as free reducing monosaccharides (e.g., for HPLC), the methyl glycoside can be removed by hydrolysis. The reagent can be obtained from commercial sources or made in laboratory by bubbling HCl gas through methanol until the desired pH is reached or by adding a molar equivalent of acetyl chloride to methanol.
2. An equilibrium of the α and β methyl glycosides of monosaccharide furanose (*f*) and pyranose (*p*) rings is achieved after 18 h so that a characteristic ratio of the four possible (fα, fβ, pα, pβ) molecules is formed to aid in unambiguous monosaccharide assignment.
3. Solid-silver carbonate has a pink hue in an acidic environment, and, therefore, neutrality can be assumed when green coloration is achieved.
4. The acidic conditions remove *N*-acetyl groups, which are replaced by acetic anhydride. This means that the original status of *N*-acetylation of hexosamines and sialic acids is not determined in the analysis procedure. If overacetylation occurs, the time can be reduced.
5. Direct re-*N*-acetylation by the addition of pyridine-acetic anhydride 1:1 in the absence of silver carbonate can be achieved, but this gives more variable results.

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Determination of Monosaccharide Linkage and Substitution Patterns by GC-MS Methylation Analysis

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

The GC or GC-MS method discussed in Chapter 130 can distinguish substituted monosaccharides, but to characterize the position of acyl groups together with the linkages between the monosaccharides, a strategy has been developed to “capture” the substitution pattern by methylation of all free hydroxyl groups. The constituent monosaccharides are then analyzed after hydrolysis, reduction, and acetylation as partially methylated alditol acetates in a procedure known as methylation analysis ***(1–3)***.

2. Materials

1. DMSO.
2. Methyl iodide.
3. Sodium hydroxide (anhydrous).
4. Chloroform.
5. Acetonitrile.
6. Pyridine.
7. Ethanol.
8. Water.
9. V-bottomed reacti-vials (Pierce [Rockford, IL]) with Teflon-backed silicone lid septa.
10. Sodium borodeuteride.
11. Ammonium hydroxide.
12. Trifluoroacetic acid.

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13. Acetic acid.
14. GC-MS (e.g., Hewlett Packard 5890/5972A with ultra-2 or HP-5MS capillary column).

3. Method

1. Dry 20 nmol pure, desalted oligosaccharide into "V"-bottomed reacti-vials in a dessicator containing P_2O_5.
2. Resuspend samples into 150 µL of a suspension of powdered NaOH/anhydrous DMSO (approx 60 mg/mL) under an inert atmosphere (*see* **Note 1**).
3. Add 75 µL of methyl iodide (care) and sonicate for 15 min (*see* **Note 2**).
4. Extract the permethylated glycans with 1 mL $CHCl_3$ 3 mL H_2O, washing the aqueous phase with 3 × 1 mL $CHCl_3$. Wash the combined $CHCl_3$ washes with 3 × 5 mL H_2O (*see* **Note 3**).
5. Dry the $CHCl_3$ phase under N_2 after taking an aliqout for liquid secondary ion mass spectrometry (LSIMS) (*see* **Note 4**).
6. Hydrolyze for 1 h in 2 *M* TFA at 100°C.
7. Reduce samples with 50 m*M* $NaBD_4$/50 m*M* NH_4OH (*see* **Note 5**) at 4°C or 4 h at room temperature.
8. Evaporate once from AcOH and three times from 1:10 AcOH/MeOH.
9. Re *N*-acetylate samples with 50:50 acetic anhydride/pyridine 100°C, 90 min.
10. Analyze samples by GC-MS on low-bleed 5% capillary column (e.g., HP ultra-2 or HP5-MS or equivalent), with either on-column or splitless injection and a temperature gradient from to 50–265°C over 21 min, held for a further 10 min, and a constant gas flow of 1 mL/min into the MS. Ionization is in EI mode and a mass range of 45–400 (*see* **Note 6**).

4. Notes

1. The NaOH can be powdered either in a mortar and pestle or glass homogenizer and thoroughly vortexed with the DMSO prior to addition to the glycan.
2. If at the end of the methylation a yellow color is present, the reaction can be stopped by adding a crystal of sodium thiosulfate with aqueous extraction.
3. The permethylated oligosaccharides may also be purified on a Sep-Pak C_{18} column (Waters, Wafford, UK) by elution of permethylated oligosaccharides with acetonitrile or acetonitrile–water mixture.
4. The reaction with the NaOH/DMSO suspension deprotonates all the free hydroxyl groups and NH of acetamido groups forming an unstable carbanion. The addition of methyl iodide then rapidly reacts with the carbanions to form O-Me groups, and thus, permethylate the oligosaccharide. When subjected to LSIMS, these permethylated oligosaccharides will fragment about their glycosidic bonds particularly at hexosacetamido residues. This means each oligosaccharide generates a unique fragmentation pattern allowing the determination of the oligosaccharide sequence. For example, the

oligosaccharide Hex-HexNAc-HexNAc-Hex will generate the following fragments: Hex-HexNAc and Hex-HexNAc-HexNAc, where Hex denotes a hexose residue and HexNAc an *N*-acetylhexosamine.

5. The reduction can also be carried out with 50 m*M* NaOH, but this is not volatile and is harder to remove prior to further derivitization.
6. The hydrolysis step generates monosaccharides with the hydroxyl groups involved in the glycosidic linkages still retaining their protons. Reduction of these monosaccharides with $NaBD_4$ will break the ring structure to form monosaccharide alditols with the anomeric (C_1) carbon being monodeuterated. Acetylation of the free hydroxyls to *O*-acetyl groups completes the derivitization. The retention times of the PMAAs on the GC allow the assignment of the monosaccharide type (galactose, *N*-acetylgalactosamine, and so on). On-line mass spectrometric detection identifies fragment ions formed by the cleavage of C—C bonds of the monosaccharide alditols with the preference: methoxy-methoxy > methoxy-acetoxy > acetoxy-acetoxy. The resulting spectra are diagnostic for the substitution pattern, and hence, the previous position of linkage, e.g., a 2-linked hexose will produce a different set of ions to a 3-linked hexose, and a 2,3-linked hexose being different again. Selected ions from the spectra of all commonly occurring linkages can be used to analyze across the chromatogram (selected ion monitoring).

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Sialic Acid Analysis by HPAEC-PAD

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1. Introduction

The most labile monosaccharides are the family of sialic acids, which are usually chain-terminating substituents. These are therefore usually released first by either mild acid hydrolysis or enzyme digestion, and can be analyzed with great sensitivity by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD ***[1, 2]***). The remaining oligosaccharide is analyzed as discussed in Chapter 131 to identify the position of linkage of the sialic acid.

2. Materials

1. HCl (0.01, 0.1, and 0.5 *M*).
2. 100 m*M* NaOH.
3. 1 *M* NaOAc.
4. HPLC and PA1 columns as described in Chapter 129.

3. Method

1. Dry the glycoprotein into a clean screw-top vial with a Teflon-backed silicone lid insert (*see* **Note 1**).
2. Release the sialic acids by hydrolysis with 0.01 or 0.1 *M* HCl for 60 min at 70°C in an inert N_2 atmosphere (*see* **Note 2**).
3. Dry down the hydrolysate, and wash three times with HPLC-grade H_2O.
4. Prepare 500 mL of 100 m*M* NaOH, 1.0 *M* sodium acetate (eluant A).
5. Prepare 500 mL of 100 m*M* NaOH (eluant B).
6. Degas eluants by bubbling helium through them in their reservoirs (*see* **Note 3**).
7. Regenerate the HPLC column in 100% eluant B for 30 min at a flow rate of 1 mL/min.
8. Equilibrate the column in 95% eluant B for 30 min at a flow rate of 1 mL/min (*see* **Note 4**).

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9. Inject approx 0.2 nmol of sialic acid onto the column, and elute using the following gradient at a flow rate of 1 mL/min:
   a. Time = 0 min; 95% eluant B.
   b. Time = 4 min; 95% eluant B.
   c. Time = 29 min; 70% eluant B.
   d. Time = 34 min; 70% eluant B.
   e. Time = 35 min; 100% eluant B.
   f. Time = 44 min; 100% eluant B.
   g. Time = 45 min; 95% eluant B; 1 mL/min.
10. Quantitate the sialic acids by comparison with known standards run on the same day.
11. When the baseline has stabilized, the system is ready for the next injection.
12. When the analyses have been completed, regenerate the column in eluant B, and flush pumps with HPLC-grade H_2O (*see* **Note 5**).

4. Notes

1. If problems with contaminants are encountered, it may be necessary to wash the vials with chromic acid overnight, wash them thoroughly with distilled water, and then treat with a hydrophobic coating, such as repelcoat (*see* Chapter 129).
2. 0.01 *M* HCl will release sialic acids with intact *N*- or *O*-acyl groups, but without quantitative release of the sialic acids. These can also be detected by HPAEC-PAD ***(3)***. At 0.1 *M* HCl, quantitive release is achieved, but with some loss of *O*- and *N*-acylation. At this concentration, some fucose residues may also be labile. Alternatively, the sialic acids can be released by neuraminidase treatment, which can be specific for α2–6 or α2–3 linkage, e.g., with α-sialidase of *Arthrobacter ureafacians* for α2–6 and α-sialidase of Newcastle disease virus for α2–3 using the manufacturer's instructions.
3. Use polypropylene reagent vessels as far as possible for HPAEC-PAD because of the corrosive nature of the NaOH, and to minimize leaching of contaminants from the reservoirs.
4. For maximum efficiency of detection, always ensure that the PAD reference electrode is accurately calibrated, the working electrode is clean, and the solvents are thoroughly degassed.
5. Failure to wash out the eluants from the pumps at the end of an analysis may result in crystallization and serious damage to the pump heads.

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Chemical Release of *O*-Linked Oligosaccharide Chains

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

O-linked oligosaccharides having the core sequences shown below can be released specifically from protein via a β-elimination reaction catalyzed by alkali. The reaction is usually carried out with concomitant reduction to prevent peeling, a reaction caused by further β-elimination around the ring of 3-substituted monosaccharides ***(1).*** The reduced oligosaccharides can be specifically bound by solid sorbent extraction on phenylboronic acid (PBA) columns ***(2).***

O-linked protein glycosylation core structures linked to Ser/Thr:

Galβ1-3GalNAcα1^{-}
GlcNAcβ1-3GalNAcα1^{-}
GlcNAcβ1-6
\\GalNAcα1^{-}
Galβ1-3/
GlcNAcβ1-6
\\GalNAcα1^{-}
GlcNAcβ1-3/
GalNAcα1-3GalNAcα1^{-}
GlcNAcβ1-6GalNAcα1^{-}
GalNAcα1-6GalNAcα1^{-}

2. Materials

1. 1 *M* $NaBH_4$ (Sigma, Poole, UK) in 50 m*M* NaOH. This is made up fresh each time from 50% (w/v) NaOH and HPLC-grade H_2O.
2. Methanol (HPLC-grade containing 1% acetic acid).
3. Acetic acid.
4. 1 mL Dowex H^+ (50 W × 12) strong cation ion-exchange column (Sigma, St. Louis, MO).

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5. Bond elute phenyl boronic acid column (Jones Chromatography, Hengoed, UK; activated with MeOH).
6. 0.1 *M* HCl.
7. 0.2 *M* NH_4OH.
8. 0.1 *M* Acetic acid.
9. Methanol.

3. Method

1. Dry the glycoprotein (100 µg to 1 mg) in a screw-topped vial and resuspend in 100 µL 50 m*M* NaOH containing 1 *M* $NaBH_4$ (*see* **Notes 1** and **2**).
2. Incubate at 55°C for 18 h.
3. Quench the reduction by the addition of ice-cold acetic acid until no further effervescence is seen.
4. Dry the reaction mixture down, and then wash and dry three times with a 1% acetic acid, 99% methanol solution to remove methyl borate.
5. Resuspend the alditols in H_2O, and pass down a 1-mL H^+ cation exchange resin. The alditols will not be retained and will elute by washing the column with water.
6. Dry the alditols and resuspend them in 100 µL of 0.2 *M* NH_4OH.
7. Activate a phenyl boronic acid (PBA) column with 2 × 1 mL MeOH.
8. Equilibrate the PBA column with 2 × 1 mL 0.1 *M* HCl, 2 × 1 mL H_2O, and 2 × 1 mL 0.2 *M* NH_4OH.
9. Add the sample in 100 µL 0.2 *M* NH_4OH and elute with 2 × 100 µL 0.2 *M* NH_4OH, 2 × 100 µL H_2O, and 6 × 100 µL 0.1 *M* Acetic acid. Collect these fractions and test for monosaccharide (Chapter 128) or combine and analyze according to Chapter 134.
10. Regenerate the PBA column with 0.1 *M* HCl and 2 × 1 mL H_2O before storing and reactivation in 2 × 1 mL MeOH.

4. Notes

1. The $NaBH_4$/NaOH solution is made up <6 h before it is required.
2. Reduction with NaB^3H_4 allows the incorporation of a radioactive label into the alditol to enable a higher degree of sensitivity to be achieved while profiling.
3. Protein degradation can be minimized by the omission of the $NaBH_4$, although this results in the degradation of sugar chains having a 3-substituted GalNAc-Ser/Thr, i.e., most types. The addition of 6 m*M* Cadmium acetate, 6 m*M* Na_2EDTA to the $NaBH_4$/NaOH solution reduces protein degradation without the loss of oligosaccharide alditol.

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O-Linked Oligosaccharide Profiling by HPLC

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

The several different core regions of *O*-linked chains (Chapter 133) can be further extended by Gal and GlcNAc containing backbones or by addition of blood group antigen-type glycosylation and the presence of sialic acid or sulfate. We sought a universal column that can be applied with high resolution to the various oligosaccharide alditols released from glycoproteins by β-elimination (*see* **Note 1**) and have pioneered ***(1, 2)*** the use of porous graphitized carbon (PGC). This is an alternative to C_{18} reversed-phase HPLC and normal-phase amino-bonded columns, which can be used together in the presence and absence of high-salt buffers ***(3)***.

2. Materials

1. Gradient HPLC system: e.g., 2 × 302 pumps, 802C manometric module, 811 dynamic mixer, 116 UV detector, 201 fraction collector, 715 chromatography system control software (all Gilson Medical Electronics, Villiers-le-Bel, France).
2. IBM PS-2 personal computer (or compatible model) with Microsoft Windows.
3. Hypercarb S HPLC Column (100 × 4.6 mm) (Shandon Scientific, Runcorn, Cheshire, England) or Glycosep H (100 × 3 mm, Oxford Glycosystems, Abingdon, UK).
4. HPLC-grade H_2O.
5. HPLC-grade acetonitrile.
6. HPLC-grade trifluoroacetic acid (Pierce and Warriner, Chester, UK).

3. Methods

1. Prepare eluant A: 500 mL of 0.05% TFA.
2. Prepare eluant B: 250 mL of acetonitrile containing 0.05% TFA.
3. Degas eluants by sparging with helium.

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4. Equilibrate the column in 100% eluant A and 0% eluant B for 30 min at 0.75 mL/min prior to injection samples.
5. Elute 10 nmol oligosaccharide alditol (obtained as in Chapter 133) with the following gradient at a flow rate of 0.75 mL/min and UV detection at 206 nm/0.08 AUFS.
    a. Time = 0 min A = 100%; B = 0%
    b. Time = 5 min A = 100%; B = 0%
    c. Time = 40 min A = 60%; B = 40%
    d. Time = 45 min A = 60%; B = 40%
    e. Time = 50 min A = 100%; B = 0%
6. The resulting oligosaccharide containing fractions are then derivatized for LSIMS and GC-MS or analyzed by NMR.

4. Note

1. The GalNAc residue linked to Ser/Thr in *O*-linked chains is normally substituted at least at C-3 and therefore the alkali catalyzed β-elimination reaction will also result in "peeling" of the released oligosaccharide. This is obviated by concomitant reduction to give oligosaccharide alditols ending in GalNAcol. Endo-α-*N*-acetylgalactosaminidase digestion at hydrazinolysis under mild conditions can be used to release intact reducing sugars, which will have longer retention times on HPLC ***(4)***.

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O-Linked Oligosaccharide Profiling by HPAEC-PAD

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

Although it can often be an advantage to be able to chromatograph neutral and sialylated oligosaccharides/alditols in one run (*see* Chapter 134), the added resolution of HPAEC and sensitivity with PAD detection means that this is an additional desirable technique for analysis. Neutral oligosaccharide alditols are poorly retained on CarboPac PA1, but can be resolved on two consecutive columns ***(1)***. The carboPac PA1 column is ideal for sialylated oligosaccharides ***(2)*** and can also be used for sialylated alditols.

2. Materials

1. Dionex D500 (Dionex, Camberley Surrey, UK) or other salt/biocompatible gradient HPLC system (titanium or PEEK) lined, e.g., 2 Gilson 302 pumps with 10-mL titanium pump heads, 802Ti manometric module, 811B titanium dynamic mixer, Rheodyne 7125 titanium injection valve with Tefzel rotor seal, and Gilson 712 chromatography system control software (Gilson Medical Electronics, Villiers-le-Bel, France).
2. CarboPac PA1 separator (4 × 250 mm) and PA1 Guard column (Dionex).
3. Pulsed amperometric detector with Au working electrode (Dionex), set up with the following parameters:
    a. Time = 0 s E = +0.1 V
    b. Time = 0.5 s E = +0.1 V
    c. Time = 0.51 s E = +0.6 V
    d. Time = 0.61 s E = +0.6 V
    e. Time = 0.62 s E = −0.8 V
    f. Time = 0.72 s E = −0.8 V
4. Anion micromembrane suppressor 2 (AMMS2) (Dionex).
5. Autoregen unit with anion regenerant cartridge (Dionex).

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6. High-purity helium.
7. NaOH 50% w/v.
8. Reagent-grade sodium acetate (Aldrich, Poole, UK).
9. HPLC-grade H_2O.
10. 500 mL of 50 m*M* H_2SO_4 (reagent-grade).

3. Methods

1. Prepare eluant A: 100 m*M* NaOH, 500 m*M* Sodium acetate.
2. Prepare eluant B: 100 m*M* NaOH.
3. Prepare the column by elution with 50% A/50% B for 30 min at a flow rate of 1 mL/min.
4. Equilibrate column is 5% eluant A/95% eluant B at a flow of 1 mL/min.
5. Connect the AMMS2 to eluant out line and autoregen unit containing 500 mL of 50 m*M* reagent-grade H_2SO_4, and pump regenerant at a flow of 10 mL/min (*see* **Note 1**).
6. Inject 200 pmol of each oligosaccharide or sialylated oligosaccharide alditol (more if required for NMR or LSIMS), and elute with the following gradient at a flow of 1 mL/min:
   a. Time = 0 min A = 5%; B = 95%
   b. Time = 15 min A = 5%; B = 95%
   c. Time = 50 min A = 40%; B = 60%
   d. Time = 55 min A = 40%; B = 60%
   e. Time = 58 min A = 0%; B = 100%
7. Equilibrate the column in 5%A/95%B prior to the next injection.
8. At the end of the analyses, regenerate the column in 100%, and flush at the pumps with H_2O.
9. Desalt oligosaccharide-containing fractions by AMMS, and derivatize for LSIMS and GC-MS.

4. Notes

1. A better desalting profile may be achieved with an AMMS membrane (rather than AMMS2) if a flow rate of <1 mL/min can be used. In addition, it is important that the membranes of the suppressor remain fully hydrated and that the regenerant solution is replaced about once a week.

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Release of *N*-Linked Oligosaccharide Chains by Hydrazinolysis

Tsuguo Mizuochi and Elizabeth F. Hounsell

1. Introduction

Hydrazinolysis is the most efficient method of releasing all classes of *N*-linked oligosaccharide chains from glycoproteins. Disadvantages compared to enzymic release (*see* Chapter 137) is the use of hazardous chemicals, break up of the protein backbone, and destruction of some chains. The first of these can be obviated by use of a commercial machine for hydrazinolysis—the Glyco Prep (Oxford GlycoSystems, Abingdon, UK), but this is expensive, and as long as caution is exercised, the following method is excellent ***(1)***.

2. Materials

1. Anhydrous hydrazine (*see* **Note 1**).
2. Toluene.
3. Saturated sodium bicarbonate solution prepared at room temperature.
4. Acetic anhydride.
5. 1-Octanol.
6. Lactose.
7. 1 *N* Acetic acid.
8. 1 *N* NaOH.
9. Methanol.
10. NaOH (0.05 *N*) freshly prepared from 1 *N* NaOH just before use.
11. Sodium borotritide (NaB^3H_4, approx 22 GBq/mmol) and approx 40 m*M* in dimethylformamide (silylation grade # 20672, Pierce Chemical Co., Rockford, IL).
12. 1-Butanol:ethanol:water (4:1:1, v/v).
13. Ethyl acetate:pyridine:acetic acid:water (5:5:1:3 v/v).
14. Dowex 50W-X12 (H^+ form, 50–100 mesh).

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15. Whatman 3MM chromatography paper.
16. Air-tight screw-cap tube with a Teflon disk seal.
17. Dry heat block capable of maintaining 100°C.
18. Vacuum desiccator.
19. High-vacuum oil pump.
20. Descending paper chromatography tank.

3. Method (*see* ref. 1)

1. Add the glycoprotein (0.1–100 mg) to an air-tight screw-cap tube with a Teflon disk seal, and dry *in vacuo* overnight in a desiccator over P_2O_5 and NaOH.
2. Add anhydrous hydrazine (0.2–1.0 mL) with a glass pipet. The pipet must be dried to avoid introducing moisture into the anhydrous hydrazine (*see* **Note 2**).
3. Heat at 100°C for 10 h using a dry heat block. Glycoprotein is readily dissolved at 100°C.
4. Remove hydrazine by evaporation *in vacuo* in a desiccator. To protect the vacuum oil pump from hydrazine, connect traps between the desiccator and the pump in the following order: cold trap with dry ice and methanol, concentration, H_2SO_4-trap, and NaOH-trap on the desiccator side. Remove the last trace of hydrazine by coevaporation with several drops of toluene.
5. To re-*N*-acetylate, dissolve the residue in ice-cold saturated $NaHCO_3$ solution (1 mL/mg of protein). Add 10 µL of acetic anhydride, mix, and incubate for 10 min at room temperature. Re-*N*-acetylation is continued at room temperature by further addition of 10 µL (three times) and then 20 µL (three times) of acetic anhydride at 10-min intervals. The total volume of acetic anhydride is 100 µL/1 mL of saturated $NaHCO_3$ solution. Keep the solution on ice until the addition of acetic anhydride to avoid epimerization of the reducing terminal sugar.
6 To desalt, pass the reaction mixture through a column (1 mL for 1 mL of the $NaHCO_3$ solution) of Dowex 50W-X12, and wash with five column bed volumes of distilled water. Evaporate the effluent to dryness under reduced pressure at a temperature below 30°C. Addition of a drop of 1-octanol is effective in preventing bubbling over.
7. Dissolve the residue in a small amount of distilled water, and spot on a sheet of Whatman 3MM paper. Perform paper chromatography overnight using 1-butanol:ethanol:water (4:1:1, v/v) as developing solvent (*see* **Note 3**).
8. Cut the area 0–4 cm from the origin, recover the oligosaccharides by elution with distilled water, and then evaporate to dryness under reduced pressure. On this chromatogram, lactose migrates <4 cm from the origin.
9. To label oligosaccharides with tritium, dissolve the oligosaccharide fraction thus obtained in 100 µL of ice-cold 0.05 *N* NaOH (freshly prepared from 1 *N* NaOH). Verify that the pH of the oligosaccharide solution is above 11 with pH test paper using a <1-µL aliquot. If not, adjust the pH paper as 0.05 *N* NaOH. After addition of NaOH solution, keep the oligosaccharide solution on ice; otherwise, part of the reducing terminal *N*-acetylglucosamine may be converted to *N*-acetylmannosamine by epimerization (*see* **Note 4**).

10. Add a 20 *M* excess of NaB^3H_4 solution to the oligosaccharide solution, mix, and incubate at 30°C for 4 h to reduce the oligosaccharides. Then, add an equal weight of $NaBH_4$ (20 mg/mL of 0.05 *M* NaOH, freshly prepared) as the original glycoprotein, and continue the incubation for an additional 1 h to reduce the oligosaccharides completely. Stop the reaction by acidifying the mixture with 1 *N* acetic acid. During the reduction, and addition of acetic acid, keep the reaction mixture in a draft chamber, since tritium gas is generated (*see* **Note 5**).
11. To desalt, apply the reaction mixture to a small Dowex 50W-X12 column, and wash with five column bed volumes of distilled water, and then evaporate the effluent under reduced pressure below 30°C. The volume of the column should be calculated based on the amount of NaOH and $NaBH_4$, and the capacity of the resin. Then, remove the boric acid by repeated (three to five times) evaporation with methanol under reduced pressure. Dimethyl formamide used to dissolve NaB^3H_4 is usually coevaporated during the repeated evaporation.
12. Dissolve the residue in a small amount of distilled water, spot on a sheet of Whatman 3MM paper, and perform paper chromatography overnight using ethyl acetate:pyridine:acetic acid:water (5:5:1:3, v/v) as developing solvent. This procedure is effective in removing the radioactive components originating from NaB^3H_4, which migrate a significant distance on the chromatogram.
13. Recover radioactive oligosaccharides, which migrate slower than lactitol (about 20 cm from origin), from the paper by elution with distilled water, and evaporate to dryness under reduced pressure.
14. Finally, subject the radioactive *N*-linked oligosaccharides thus obtained to high-voltage paper electrophoresis at pH 5.4 or HPLC to separate oligosaccharides by charge.

4. Notes

1. Anhydrous hydrazine is prepared by mixing 80% hydrazine hydrate (50 g), toluene (500 g), and CaO (500 g) and allowing to stand overnight. The mixture is refluxed for 3 h using a cold condenser and an NaOH tube. The mixture is then subjected to azeotropic distillation with toluene at 93–94°C under anhydrous conditions. Anhydrous hydrazine is collected from the bottom layer and stored in an air-tight screw-cap tube with a Teflon disk seal under dry conditions at 4°C in the dark. Commercially available anhydrous hydrazine (such as that from Aldrich Chemical Co., Inc., Milwaukee, WI) can also be used. It is important to check the quality with a glycoprotein of which the oligosaccharide structure has already been established before using for analysis, because contamination by trace amounts of water in some lots could modify the reducing terminal *N*-acetylglucosamine of *N*-linked oligosaccharides.

   Caution: Anhydrous hydrazine is a strong reducing agent, highly toxic, corrosive, suspected to be carcinogenic, and flammable. Therefore, great caution should be exercised during handling.

2. When stored in a small air-tight tube with a Teflon disk seal at −18°C, this NaB^3H_4 solution is stable for at least 1 yr. Dimethylformamide should be stored with molecular sieves in a small screw-cap bottle with a Teflon disk seal under dry conditions.
3. This procedure is indispensable for the next tritium-labeling step of the liberated oligosaccharides. This is because oligosaccharides larger than trisaccharides remain very close to the origin, whereas the degradation products derived from the peptide moiety, which react with NaB^3H_4, move a significant distance on the paper.
4. For quantitative liberation of intact *N*-linked oligosaccharides from glycoproteins by hydrazinolysis, great care should be taken to maintain anhydrous condition until the re-*N*-acetylation step. Introduction of moisture into glycoprotein samples or anhydrous hydrazine results in diverse modifications of reducing terminal *N*-acetylglucosamine residues, especially when unsubstituted with an Fucα 1–6 group, and causes the release of *O*-linked oligosaccharides accompanied with various degradations of the reducing end.
5. When oligosaccharides are reduced by NaB^3H_4 with high specific activity (e.g., 555 GBq/mmol), the sensitivity of detection of oligosaccharides increases about 20-fold. To label oligosaccharides with tritium at high efficiency, it is recommended to keep the concentration of NaB^3H_4 high in the incubation mixture by reducing the volume of 0.05*N* NaOH (e.g., to the same volume as the NaB^3H_4 solution). A 20 *M* excess of NaB^3H_4 solution is required for complete reduction of *N*-linked oligosaccharides, whereas a 5 *M* excess of NaB^3H_4 solution required for complete reduction of *N*-linked oligosaccharides derived from glycoprotein samples is roughly estimated from data on the carbohydrate content or amino acid sequence. If generation of tritium gas is to be avoided, continue the incubation for an additional 1 h with large amounts of glucose to absorb excess NaB^3H_4 before acidifying the mixture.

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Enzymatic Release of *O*-and *N*-Linked Oligosaccharide Chains

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

Enzymes involved in both the synthesis and degradation of glycoconjugates are highly specific for monosaccharide, linkage position, and anomeric configuration factors further away in the oligosaccharide sequence or protein. Not withstanding this, endo- and exoglycosidases are extremely useful tools in structural analysis. The RAAM technique has automated the use of exoglycosidase digestion (Oxford Glycosystems, Abingdon, UK). Here we discuss the release of intact oligosaccharide chains from proteins that can be further analyzed for separate functions ***(1,2)***.

2. Materials

2.1. Desalting

1. mL Spectra/Chrom desalting cartridge (Orme, Manchester, UK) or Biogel P_{-2} minicolumn.
2. HPLC-grade H_2O.

2.2. Glycosidases

1. Endoglycosidase H (EC 3.2.1.96) (e.g., *E. coli,* Boehringer Mannheim, Lewes, UK). Digestion buffer: 250 m*M* sodium citrate buffer adjusted to pH 5.5 with 1 *M* HCl.
2. Test-neuraminidase (EC 3.2.1.18) (e.g., *Vibrio cholerae,* Behring Ag, Marburg, Germany). Made up as 1 U/mL enzyme in digestion buffer and stored at 4°C. Digestion buffer: 50 m*M* sodium acetate, 134 m*M* NaCl, 9 m*M* $CaCl_2$.
3. Peptide-*N*-glycosidase F (EC 3.2.2.18) (e.g., *Flavobacterium meningosepticum,* Boehringer Mannheim). Digestion buffer: 40 m*M* potassium dihydrogen orthophosphate (KH_2PO_4), 10 m*M* EDTA adjusted to pH 6.2 with 1.0 *M* NaOH.

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4. *O*-glycosidase (EC 3.2.1.97) (e.g., *Diplococcus pneunomiae,* Boehringer Mannheim). Digestion buffer: 40 m*M* KH_2PO_4/10 m*M* EDTA adjusted to pH 6.0 with 1.0 *M* NaOH.
5. Ice-cold ethanol.
6. Toluene.

3. Methods

3.1. Desalting

1. Wash the cartridge with 5 mL of HPLC-grade H_2O.
2. Load the sample onto the cartridge in a volume between 50 and 200 µL H_2O.
3. Elute the column with 200 µL of H_2O (including sample load).
4. Elute the glycoprotein in 350 µL of H_2O.
5. Elute the salt with an additional 1 mL of H_2O.

3.2. Glycosidase Digestions

1. Dissolve 1 nmol of glycoprotein in 100 µL of H_2O, and boil for 30 min to denature. Remove a 10% aliquot for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as a control for detection of enzyme digestion. Dry the remainder by lyophilization (*see* **Note 1**).
2. Resuspend the glycoprotein in 500 µL of Endo H digestion buffer and 5 µL of toluene. Add 1 mU of Endo H/l nmol glycoprotein, and incubate at 37°C for 72 h (*see* **Notes 2** and **3**).
3. Precipitate the protein with a twofold excess of ice-cold ethanol and centrifuge at 15,000g for 20 min. Wash the pellet three more times with ice-cold ethanol. Dry the protein pellet, take up in water, and aliquot 10% (relative to original amount) for SDS-PAGE.
4. Dry the remaining pellet, and resuspend in neuraminidase/neuraminidase digestion buffer at a concentration of 2 nmol of glycoprotein/10 µL of buffer. Incubate for 18 h at 37°C, and then ethanol-precipitate and aliquot as in **step 3**.
5. Resuspend the remaining glycoprotein in 500 µL of PNGase F digestion buffer, 5 µL toluene, and 1 U PNGase F/10 nmol glycoprotein. Incubate at 37°C for 72 h before precipitation and aliquoting as in **step 3** (*see* **Note 4**).
6. Digest the final pellet with *O*-glycosidase under the same conditions as for the PNGase F digestion. Precipitate the pellet from ethanol washing and dry.
7. Apply all the pellet to SDS-PAGE.
8. The supernatants containing *N*- and *O*-linked oligosaccharides can be analyzed as discussed in Chapters 111, 118, and 119.

4. Notes

1. The described procedure assumes approx 10% glycosylation of the glycoprotein. The amount of glycoprotein treated may have to be increased to obtain oligosaccharides for further analysis with less highly glycosylated glycoproteins.

2. PNGase F is stored at –20°C, and all other enzymes at 4°C. Endo H removes high-mannose oligosaccharide chains, but not complex type.
3. The toluene is added to prevent bacterial growth.
4. The PNGase F digestion can be performed directly on the boiled glycoprotein if all *N*-linked glycoprotein chains (both high mannose and complex) are required to be removed. Digests may also be performed in 0.2 *M* sodium phosphate buffer, pH 8.4, but this will result in the release of sialic acid residues as monosaccharides.

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N-Linked Oligosaccharide Profiling by HPLC on Porous Graphitized Carbon (PGC)

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

The vast array of possible *N*-linked oligosaccharides demands high-resolution HPLC columns for their purification ***(1,2)***. Reverse-phase (C_{18}) and normal-phase (NH_2) columns have been used for the separation (singly or in concert) of many *N*-linked oligosaccharides. The porous graphitized carbon (PGC) column described in Chapter 134 for *O*-linked alditol separation will give improved *N*-linked oligosaccharide resolution over C_{18} columns, and has the advantage of using salt-free buffers for preparative work ***(3,4)***.

2. Materials

1. Biocompatible gradient HPLC system, e.g., 2 × 302 pumps, 802C manometric module, 811 dynamic mixer, 116 UV detector, 201 fraction collector, 715 chromatography system control software (all Gilson Medical Electronics, France).
2. IBM PS-2 personal computer (or compatible model) with Microsoft Windows.
3. Hypercarb S HPEC Column (100 × 4.6 mm) (Shandon Scientific, Runcorn, Cheshire, England) or Glyco H (OGS, Abingdon, Oxon, UK).
4. HPLC-grade H_2O.
5. HPLC-grade acetonitrile.
6. HPLC-grade trifluoroacetic acid (Pierce and Warriner, Chester, UK).

3. Methods

1. Prepare eluant A: 500 mL of 0.05% TFA.
2. Prepare eluant B: 250 mL of acetonitrile containing 0.05% TFA.
3. Degas eluants by sparging with helium.
4. Equilibrate the column in 100% eluant A, and 0% eluant B for 30 min at 0.75 mL/min prior to injection of samples.

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5. Elute 10 nmol oligosaccharides with the following gradient at a flow rate of 0.75 mL/min and UV detection at 206 nm/0.08 AUFS (*see* **Note 1**).
   a. Time = 0 min A = 100%; B = 0%
   b. Time = 5 min A = 100%; B = 0%
   c. Time = 40 min A = 60%; B = 40%
   d. Time = 45 min A = 60%; B = 40%
   e. Time = 50 min A = 100%; B = 0%
6. The resulting oligosaccharide containing fractions are then derivatized for LSIMS and GC-MS or analyzed by NMR.

4. Notes

1. Reverse-phase or normal-phase HPLC may also be required for the complete separation of some oligosaccharide isomers.
2. To prevent anomerization, the oligosaccharides can be reduced to their alditols (either after PNGase F digestion or hydrazinolysis). The inclusion of a ^{3}H-label on reduction will give increased sensitivity over UV. Alternatively, the oligosaccharides can be fluorescently labeled at their reducing terminus (with 2-amino-benzamide or 2-amino-pyridine) by reductive amination to give a fluorescent chromophore and increased sensitivity. Sensitivity of detection may also be increased by the postcolumn addition of 300 m*M* NaOH and pulsed amperometric detection as described for HPAEC-PAD.

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N-Linked Oligosaccharide Profiling by HPAEC-PAD

Elizabeth F. Hounsell, Michael J. Davies, and Kevin D. Smith

1. Introduction

High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is a very powerful tool for the profiling of *N*-linked oligosaccharides ***(1, 2)***, only being limited by the concentrated sodium hydroxide and sodium acetate required to achieve the separation and sensitive detection. Oligosaccharides are separated on the basis of charge (i.e., the number of sialic acid residues) and the linkage isomers present. Neutral oligosaccharide alditols are most weakly retained, retention increasing with increasing sialylation, and NeuGc- and sulfate-bearing oligosaccharides being most strongly retained. Fucosylation results in a shorter retention. If the oligosaccharides can be effectively desalted after HPAEC-PAD, this remains the method of choice for oligosaccharide purification, and is a very powerful analytical tool.

2. Materials

1. Dionex DX300 or salt/biocompatible gradient HPLC system (titanium or PEEK lined), e.g., 2 Gilson 302 pumps with 10-mL titanium pump heads, 802Ti manometric module, 811B titanium dynamic mixer, Rheodyne 7125 titanium injection valve with Tefzel rotor seal, and Gilson 715 chromatography system control software (all Gilson Medical Electronics, Villiersle-Bel, France).
2. IBM PS-2 personal computer (or compatible model) with Microsoft Windows.
3. CarboPac PA100 separator (4 × 250 mm) and PA100 guard column (Dionex, Camberley, UK).
4. Pulsed amperometric detector with Au working electrode (Dionex), set up with the following parameters:

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   a. Time = 0 s E = +0.1 V
   b. Time = 0.5 s E = +0.1 V
   c. Time = 0.51 s E = +0.6 V
   d. Time = 0.61 s E = +0.6 V
   e. Time = 0.62 s E = –0.8 V
   f. Time = 0.72 s E = –0.8 V

5. High-purity helium.
6. NaOH 50% (w/v).
7. ACS-grade sodium acetate (Aldrich, UK).
8. HPLC-grade H_2O.

3. Methods

1. Prepare eluant A: 100 m*M* NaOH, 500 m*M* sodium acetate.
2. Prepare eluant B: 100 m*M* NaOH.
3. Prepare the column by elution with 50% A/50% B for 30 min at a flow rate of 1 mL/min.
4. Equilibrate column in 5% eluant A/95% eluant B at a flow of 1 mL/min.
5. Inject 200 pmol of each oligosaccharide or sialylated oligosaccharide alditol (more if required for NMR or LSIMS) and elute with the following gradient at a flow of 1 mL/min:
   a. Time = 0 min A = 5%; B = 95%
   b. Time = 15 min A = 5%; B = 95%
   c. Time = 50 min A = 40%; B = 60%
   d. Time = 55 min A = 40%; B = 60%
   e. Time = 58 min A = 0%; B = 100%
6. Equilibrate the column in 5%A/95%B prior to the next injection.
7. At the end of the analyzes, regenerate the column in 100%B, and flush the pumps with H_2O.
8. Desalt oligosaccharide-containing fractions and derivatize for LSIMS, GC-MS, or NMR (*see* **Note 1**).

4. Notes

1. Desalting can either be achieved by means of a ion-suppression system (e.g., Dionex AMMS or SRS system) or by off-line desalting on Biogel P2 mini-columns or H^+ cation-exchange resins. Fractions containing multiply sialylated oligosaccharides will probably contain too much salt to be totally desalted by an ion-suppressor, and column methods will be required. This can lead to losses of minor oligosaccharides.
2. All previous notes for HPAEC-PAD methods in this volume also apply.

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HPAEC-PAD Analysis of Monosaccharides Released by Exoglycosidase Digestion Using the CarboPac MA1 Column

Michael Weitzhandler, Jeffrey Rohrer, James R. Thayer, and Nebojsa Avdalovic

1. Introduction

Exoglycosidases are useful reagents for the structural determination of glycoconjugates. Their anomeric, residue, and linkage specificity for terminal monosaccharides have been used to assess monosaccharide sequence and structure in a variety of glycoconjugates *(1)*. Their usefulness depends on the absence of contaminating exoglycosidases and an understanding of their specificity. Digestions of oligosaccharides with exoglycosidases give two classes of products: monosaccharides and the shortened oligosaccharides. Most assays of such reactions have monitored the reaction by following oligosaccharides that are labeled at their reducing ends. In these assays, after exoglycosidase digestion the shortened oligosaccharide retains the label at its reducing end. The other digestion product, the released monosaccharide, does not carry a label and thus cannot be quantified. Additionally, identification of any other monosaccharide that could be the result of a contaminating exoglycosidase activity would not be possible. Quantitative measurement of all products (all released monosaccharide[s] as well as the shortened oligosaccharide product) would be useful because it would enable the determination of any contaminating exoglycosidase activities by determining the extent of release of other monosaccharides. High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) detects the appearance of monosaccharide product(s), the shortened oligosaccharide product(s) as well as the disappearance of the oligosaccharide substrate(s) in a single chromatographic analysis without labeling. Thus, HPAEC-PAD has been used extensively to monitor the activities of several different exoglycosidases

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on glycoconjugates, usually using the CarboPac PA1 column to separate the digestion products (*see* **refs.** *1–8*).

A problem encountered when using HPAEC-PAD to monitor exoglycosidase digestions is that *N*-acetylglucosamine (GlcNAc) is not baseline resolved from other glycoconjugate monosaccharides on the CarboPac PA1 column using the isocratic conditions that successively baseline resolve glycoprotein hydrolysis products: fucose, galactosamine, glucosamine, galactose, glucose, and mannose ***(9)***. We recently discovered that the CarboPac MA1 column, developed for the separation of neutral sugar alditols ***(10,11)***, gives an isocratic baseline separation of GlcNAc, GalNAc, fucose, mannose, glucose, and galactose, and simultaneously resolves many neutral oligosaccharides. This separation extends the usefulness of the CarboPac MA1 column to the assay of reducing monosaccharides released by exoglycosidases. In the following, an assay of exposed GlcNAc after β-*N*-acetylhexosaminidase treatment of a variety of glycoconjugates is shown.

To determine the suitability of HPAEC-PAD and the MA1 column for analyzing both released monosaccharide and oligosaccharide products in a single analysis, we subjected an asialo agalacto biantennary oligosaccharide standard (**Table 1**, structure 3; **Fig. 1A**, peak 3) and an asialo agalacto tetraantennary oligosaccharide standard (**Table 1**, structure 5; **Fig. 1B**, peak 5) to Jack bean β-*N*-acetylhexosaminidase digestion. In addition to differences in numbers of antennae (2 vs 4) and retention times (22.3 vs 25.2 min), these two oligosaccharides differ in that the tetraantennary oligosaccharide has terminal GlcNAc linked to mannose β(1→4) and β(1→6) in addition to the β(1→2) linkages present in the biantennary oligosaccharide standard.

The complete disappearance of the asialo agalacto biantennary substrate is indicated by the disappearance of peak 3 (**Fig. 1A**; compare dashed vs solid line in bottom tracing). The complete disappearance of the asialo agalacto tetraantennary substrate is indicated by the disappearance of peak 5 (**Fig. 1B**; compare dashed vs solid line in bottom tracing). The expected digestion products of both structures 3 and 5 would be the released monosaccharide GlcNAc, (*see* **Fig. 1A,B**; peak at 15.6 min) and the released, shortened oligosaccharide product, $Man_3GlcNAc_2$ (**Table 1**, structure 1; *see* **Fig. 1A,B**, peak at 17.5 min).

In addition to being useful for monitoring the β-*N*-acetylhexosaminidase release of terminal GlcNAc from isolated oligosaccharides, HPAEC-PAD and the MA1 column can be used to directly monitor the presence of terminal GlcNAc on glycoproteins. Such an assay could be useful for monitoring terminal carbohydrate modifications in therapeutic glycoproteins; these modifications have been shown to affect the stability and efficacy of therapeutic glycoproteins ***(12)***. The β-*N*-acetylhexosaminidase release of terminal GlcNAc from a monoclonal IgG is shown in **Fig. 2A** (bottom tracing; compare solid vs dashed line). Additionally, the absence of contaminating exoglycosidases is apparent by the absence of release of any other monosaccharides.

Table 1
Oligosaccharide Substrates for Exoglycosidase Analyses

Structure	Retention Time
1. *Man$_3$GlcNAc$_2$*: Manα(1→6) ↓ Manβ(1→4)GlcNAcβ(1→4)GlcNAc ↑ Manα(1→3)	17.5 min.
2. *Fucosylated Man$_3$GlcNAc$_2$*: Manα(1→6) Fucα(1→6) ↓ ↓ Manβ(1→4)GlcNAcβ(1→4)GlcNAc ↑ Manα(α1→3)	13.7 min
3. *Asialo agalacto biantennary*: GlcNAcβ(1→2)Manα(1→6) ↓ Man(β1→4)GlcNAcβ(1→4)GlcNAc ↑ GlcNAcβ(1→2)Manα(1→3)	22.3 min.
4. *Asialo agalacto biantennary, Core Fuc*: GlcNAcβ(1→2)Manα(1→6) Fucα(1→6) ↓ ↓ Man(β1→4)GlcNAcβ(1→4)GlcNAc ↑ GlcNAcβ(1→2)Manα(1→3)	18.7 min.
5. *Asialo agalacto tetraantennary*: GlcNAcβ(1→6) ↓ GlcNAcβ(1→2)Manα(1→6) ↓ Man(β1→4)GlcNAcβ(1→4)GlcNAc ↑ GlcNAcβ(1→2)Manα(1→3) ↑ GlcNAcβ(1→4)	25.2 min.

To assess whether the released GlcNAc was derived from a GlcNAc-terminated *N*-linked oligosaccharide, the monoclonal IgG was treated with peptide-*N*-glycosidase (PNGase F), an amidase that nonspecifically releases *N*-linked oligosaccharides from glycoproteins. The *N*-linked oligosaccharide map of the monoclonal IgG is shown in **Fig. 2B** (bottom tracing; solid line).

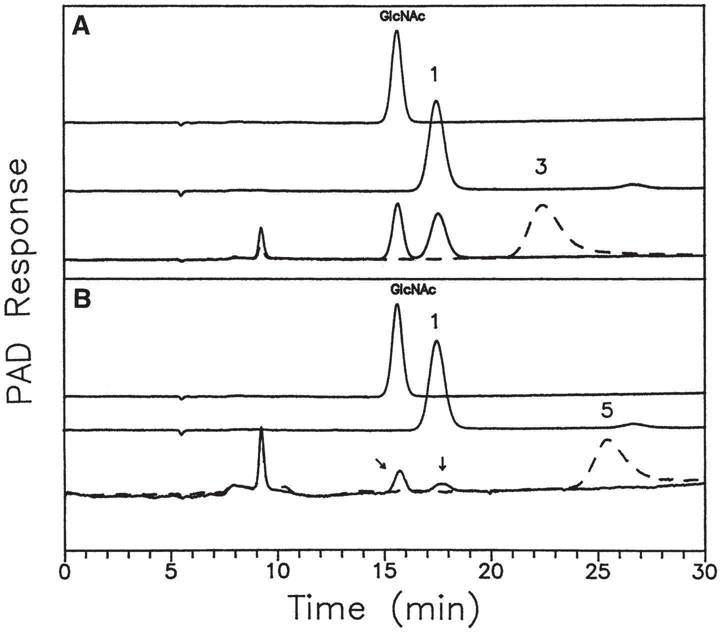

Fig. 1. HPAEC-PAD of β-*N*-acetylhexosaminidase digests of oligosaccharide standards. **(A)** Digestion of an asialo agalacto biantennary oligosaccharide standard. **(B)** Digestion of an asialo agalacto tetraantennary oligosaccharide standard. Peaks 1, 3, and 5 refer to structures 1, 3, and 5 identified in **Table 1**. (Bottom tracings in **A** and **B**: dashed line, chromatography of substrate; solid line, chromatography of digestion products.)

The major PNGase F product peak eluted between 18.5 and 19 min with a retention time similar to a core fucosylated asialo agalacto biantennary oligosaccharide standard (18.7 min, *see* **Table 1**, structure 4; *see also* **Fig. 2B**, peak 4). The second PNGase F released peak had a retention time of 21 min and could represent a monogalactosylated, biantennary oligosaccharide with core fucosylation, as has been reported in other monoclonal IgGs *(7)*. To assess whether the PNGase F released oligosaccharides were terminated with GlcNAc, the PNGase F released oligosaccharides were treated with β-*N*-acetylhexosaminidase.

The results of β-*N*-acetylhexosaminidase treatment of the PNGase F released oligosaccharides are depicted in **Fig. 2B** (second from bottom tracing). Both peaks corresponding to PNGase F released oligosaccharides disappeared and two new peaks appeared. The two new peaks had retention times corresponding to released GlcNAc (15.6 min) and to a fucosylated $Man_3GlcNAc_2$ standard (17.5 min, *see* **Table 1**, structure 2; *see also* **Fig. 2B**, peak 2). Thus, the two *N*-linked oligosaccharide peaks released from the monoclonal IgG are fucosylated and have terminal GlcNAc. To obtain further structural information

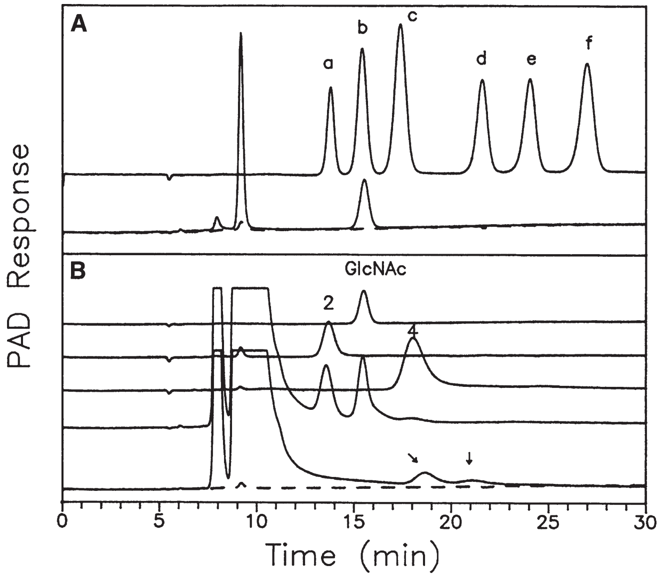

Fig. 2. HPAEC-PAD of β-*N*-acetylhexosaminidase digests of a monoclonal IgG **(A)** Bottom tracing is β-*N*-acetylhexosaminidase digests of a monoclonal IgG (dashed line, chromatography of IgG substrate; solid line, chromatography of digestion products). Top tracing is chromatography of monosaccharide standards; Peak a, fucose; b, GlcNAc; c, GalNAc; d, mannose; e, glucose; f, galactose. **(B)** Bottom tracing is PNGase F digest of a monoclonal IgG (dashed line, chromatography of IgG substrate; solid line, chromatography of PNGase F-released *N*-linked oligosaccharides from the monoclonal IgG; arrows indicate oligosaccharide peaks). β-*N*-acetylhexosaminidase treatment of PNGase F-released *N*-linked oligosaccharides is shown in the second to bottom tracing. Note appearance of product peaks corresponding to GlcNAc and fucosylated $Man_3GlcNAc_2$. Peaks 2 and 4 represent chromatography of oligosaccharide standards corresponding to structures 2 and 4 in **Table 1**.

regarding GlcNAc linkages, one could use the *Streptococcus pneumoniae* β-*N*-acetylhexosaminidase, an exoglycosidase which shows much more efficient cleavage of the GlcNAcβ(1 → 2)Man if the Man residue is not substituted with GlcNAc at the C-6 *(13)*. This method is also useful for monitoring the β-*N*-acetylhexosaminidase preparation for contaminating exoglycosidase activities as other monosaccharide digestion products would be readily discernible.

2. Materials

1. HPLC grade deionized water (*see* **Note 1**).
2. 50% NaOH solution (w/w) (Fisher Scientific, Pittsburgh, PA) (*see* **Note 2**).
3. Reference-grade monosaccharides (Pfanstiehl Laboratories, Waukegan, IL).
4. Oligosaccharide standards $Man_3GlcNAc_2$ (**Table 1**, structure 1) fucosylated $Man_3GlcNAc_2$ (**Table 1**, structure 2), asialo agalacto biantennary (**Table 1**, structure 3),

asialo agalacto biantennary, core fucose (**Table 1**, structure 4), and asialo agalacto tetraantennary oligosaccharide (**Table 1**, structure 5) (Oxford GlycoSciences, Abingdon, UK).

5. β-*N*-acetylhexosaminidase, Jack bean (Oxford GlycoSciences) (*see* **Note 3**).
6. PNGase F (New England BioLabs, Beverly, MA) (*see* **Note 4**).
7. 25-mL Plastic pipets (Fisher Scientific).
8. 1.5-mL Polypropylene microcentrifuge tubes, caps, and O rings (Sarstedt, Newton, NC).
9. Autosampler vials: 12- × 32-mm disposable, limited-volume sample vials, Teflon/silicone septa, and caps (Sun Brokers, Wilmington, NC).
10. Nylon filters (Gelman Sciences, Ann Arbor, MI).
11. The chromatograph (Dionex, Sunnyvale, CA) consists of a gradient pump, a PAD II or PED, and an eluent degas module (EDM). The EDM is used to sparge and pressurize the eluents with helium. The system was controlled and data were collected using Dionex AI450 software. Sample injection was accomplished with a Spectra Physics SP8880 autosampler (Fremont, CA) equipped with a 200-µL sample loop. The Rheodyne (Cotati, CA) injection valve is fitted with a Tefzel rotor seal to withstand the alkalinity of the eluents. We also used a DX 500 BioLC system (Dionex) configured for carbohydrate analysis with PeakNet software (Dionex).

3. Methods

3.1. β-N-Acetylhexosaminidase Digestion

1. Reconstitute approx 2 µg each of the neutral agalacto biantennary and agalacto tetraantennary oligosaccharides (**Table 1**, structures 3 and 4, respectively) in 10 µL of 25 m*M* sodium citrate-phosphate buffer, pH 5.0.
2. Add 0.1 U of Jack bean β-*N*-acetylhexosaminidase in 2 µL of 25 m*M* sodium citrate-phosphate buffer, pH 5.0.
3. Incubate the digest for 20 h at 37°C.
4. Inject 10 µL of each digest directly onto the column.

3.2. PNGase F Digestion

1. Reconstitute approx 100 µg of a monoclonal IgG in 10 µL of 25 m*M* sodium citrate-phosphate buffer, pH 5.0.
2. Add 2 µL of the PNGase F preparation.
3. To an identifical PNGase F digest of the same monoclonal IgG, add 0.1 U of Jack bean β-*N*-acetylhexosaminidase in 2 µL of 25 m*M* sodium citrate-phosphate buffer, pH 5.0.
4. Separately inject 10 µL of each digest directly onto the column.

3.3. Chromatography and Detection of Carbohydrates

Separations of monosaccharides and oligosaccharides can be achieved using a Dionex BioLC system equipped with a CarboPac MA1 column (4 × 250 mm) and a CarboPac MA1 guard column working at an isocratic concentration of

480 m*M* NaOH and a flow rate of 0.4 mL/min at ambient temperature over 35 min. Separated mono- and oligosaccharides are detected by PAD with a gold electrode and triple-pulse amperometry (E_1 = 0.05 V, t_1 = 420 ms; E_2 = 0.80 V, t_2 = 360 ms; E_3 = –0.15 V, t_3 = 540 ms), measuring at 1000 nA full scale. Alternatively, a more recently described quadruple potential waveform can be used (Dionex Technical Note 21; Waveform A). (E1 = +0.1 V, t1 = 400 ms. The first 200 ms is t del and the second 200 ms is t det; E2 = –2.0 V, t2 = 10 ms; E3 = +0.6 V, t3 = 40 ms; E4 = –0.1 V, t4 = 60 ms).

4. Notes

1. It is essential to use high quality water of high resistivity (18 MeΩ) and to have as little dissolved carbon dioxide in the water as possible. Biological contamination should be absent. The use of fresh Pyrex glass-distilled water is recommended. The still should be fed with high-resistivity (18 MeΩ) water. The use of plastic tubing in the system should be avoided, as plastic tubing often supports microbial growth. Degas appropriately.
2. It is extremely important to minimize contamination with carbonate. Carbonate, a divalent anion at pH ≥ 12, binds strongly to the columns and interferes with carbohydrate chromatography. Thus carbonate is known to affect column selectivity and produce a loss of resolution and efficiency. Commercially available NaOH pellets are covered with a thin layer of sodium carbonate and should NOT be used. Fifty percent (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for NaOH. Diluting 104 mL of a 50% NaOH solution into 2 L of water yields a 1.0 *M* NaOH solution. Degas appropriately.
3. This enzyme has a broad specificity, cleaving nonreducing terminal β-*N*-acetylglucosamine residues (GlcNAc) and β-*N*-acetylgalactosamine (GalNAc) with 1–2,3,4, and 6 linkages.
4. PNGase F is an amidase from *Flavobacterium meningosepticum*. The enzyme cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins.

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Microassay Analyses of Protein Glycosylation

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1. Introduction

The majority of secreted and cell surface proteins are glycosylated. To characterize accurately the carbohydrate moieties of oligosaccharide chains in glycosylated proteins, it is necessary to distinguish exactly which types of oligosaccharide are present and at which sites in the protein. The goal of the microassays presented here is to determine oligosaccharide structure and occupancy using lectin overlay assays. Lectins are multivalent binding proteins with the ability to bind specifically to certain sugar sequences in oligosaccharides and glycopeptides ***(1)***. Lectin overlay assays take advantage of the ability of lectins to distinguish between different types of glycoproteins by virtue of their ability to recognize terminal sugars, thus allowing the chain type and peripheral antigenic components to be determined. In addition, the assay is important as it provides structural information of protein glycosylation prior to conformational analysis and identification of antigenically and biologically active oligosaccharides. The lectin overlay, in the form of a microassay, can be developed to give a high level of sensitivity for glycan detection. The specificities of the lectins in such assays appears to vary from previous studies using lectin affinity chromatography and hence must be characterized further.

Three microassays are standardized here: The first is the analysis of non-protease-treated intact glycoproteins blotted onto nitrocellulose membranes (NCMs). Second, glycopeptides are released by prior digestion of the glycoprotein with a suitable protease that will cleave it into glycopeptides of ideally 5–15 amino acids and then separated by high performance liquid chromatography (HPLC) prior to lectin assay. Glycopeptides do not usually adhere to gels or membranes and therefore the latter need to be modified. Third, oligosaccharide chain structure has been characterized by the release of sugars from glycoproteins by endoglycosidase

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digestion or hydrazinolysis and coupling them to a multivalent support followed by lectin overlay analysis. Coupling of oligosaccharides is achieved by reductive amination. This reaction takes advantage of the availability of reducing sugars in the open chain aldehydo form for reductive amination with primary amino groups, for example, εNH_2 of lysine in proteins. The base is stabilized by Schiff base formation via reduction in sodium cyanoborohydride. We have used this method, for example, to characterize the structure and antigenicity of a series of reducing *O*-linked oligosaccharides released from pigeon mucin ***(2)***.

2. Materials

1. Glycorelease *N*- and *O*-glycan recovery kit (Oxford GlycoSystems; Abingdon, UK).
2. Porous graphitized carbon (PGC) column (Hypersil Hypercarb, 100 mm × 4.6 mm, 7 µm, Hypersil; Runcorn, UK).
3. Reagents used in reverse-phase (RP)-HPLC (*see* **Subheading 3.2.**): acetonitrile and trifloroacetic acid (TFA) (Merck & Co.; Hoddesdon, UK).
4. Rat Thy-1 protein obtained by immunoaffinity purification and the lipid moiety removed by phospholipase C ***(3)***.
5. Pigeon intestinal mucin obtained from the intestines of freshly killed pigeons by CsCl density gradient ***(4)***.
6. Bovine fetuin, asialylated bovine fetuin, bovine serum albumin, chicken ovalbumin, ExtraAvidin peroxidase conjugate, 4-chloro-1 naphthol, poly-L-lysine, ammonium carbonate, and all reagents used in the modification and activation of NCM (*see* **Subheading 3.4.**): divinyl sulfone, dimethylformamide, sodium hydrogen carbonate, disodium carbonate, ethylenediamine, glutaraldehyde, and methanol (Sigma; Poole, UK).
7. Bovine pancreatic RNase B (Oxford GlycoSystems; Abingdon, UK).
8. Immobilon-P nitrocellulose membranes (0.2-µm and 0.45-µm pore size) (Millipore Corp.; Bedford, MA, USA).
9. Digoxigenin-labeled lectins of the glycan differentiation kit (Boehringer Mannheim; Mannheim, Germany).
10. Biotinylated lectins (Vector Labs, Peterborough, UK).
11. Benchmark microplate reader (Bio-Rad Lab. Ltd.; Hemel Hempstead, UK).

3. Methods

All procedures are carried out at room temperature unless otherwise stated.

3.1. Hydrozinolysis for Release of N- and O-Linked Oligosaccharides (e.g., O-Linked Glycoproteins) Using the Glycorelease Kit

1. Directly prior to hydrazinolysis, lyophilize glycoproteins overnight in a screw-capped) vial and store in a desiccator in the presence of phosphorus pentoxide for 24 h.
2. Add 5 mL of anhydrous hydrazine to the vial, seal, and heat in a heating block at 60°C for 4 h for *O*-linked glycans (95°C for 5 h for *N*-linked glycans).

3. Cool the reaction vessel to room temperature and remove excess hydrazine by centrifugal evaporation under reduced pressure (*see* **Note 1**).
4. Acetylate the released oligosaccharides by addition of 0.5 mL of ice-cold acetylation buffer and 50 µL of acetylating reagent (supplied with the kit). Incubate mixture for 20 min at 4°C and then 30 min at room temperature.
5. Desalt oligosaccharides on a cation-exchange resin and lyophilize using a centrifugal evaporator.
6. Dissolve dried samples in a minimal amount of pure water. Purify the released glycans from peptide material by ascending paper chromatography using high-purity butanol–ethanol–water (16:4:1 by vol). Elute oligosaccharides at and a few centimeters in front of the origin using water. Filter through a 0.2-µm PTFE filter. Evaporate to dryness and finally dissolve in 100 µL of water and store at –80°C.

3.2. Glycopeptide Preparation and RP-HPLC on Porous Graphitized Carbon Column

3.2.1. N*-Linked Sugars*

1. Dissolve 1 mg of trypsin in 500 µL of digestion buffer (500 m*M* NH_4CO_3, pH 8.5) immediately before it is required.
2. Dissolve either native or reduced carboxymethylated glycoprotein in enzyme–buffer solution at 20 µg of enzyme/1 mg of glycoprotein with 5 µL of toulene to prevent bacterial growth. Incubate for up to 72 h at 37°C, with an addition of enzyme (10 µg of enzyme/1 mg of glycoprotein) after 24 h.
3. Wash the digest 3× with 0.1 mL of water.
4. Lyophilize the digest by evaporation prior to chromatography.
5. Redissolve dried sample in 0.1% aqueous TFA and inject onto an HPLC column of PGC ***(5)***.
6. Run a starting eluent for 2 min at 2% buffer A (0.1% TFA in acetonitrile) in buffer B (0.1% aqueous TFA) followed by a linear gradient to 82% buffer A for 78 min at a flow rate of 1 mL/min.
7. Collect 2-mL fractions up to 70 min and then lyophilize each fraction. Spot samples onto NCM modified to bind peptides and glycopeptides as described in **Subheading 1.4.1** (adapted from the method of **ref.** *6*) (*see* **Notes 2** and **3**).

3.2.2. O*-Linked Sugars*

1. Lyophilize *O*-linked sugars released by hydrazinolysis (*see* **Subheading 3.1**).
2. Redissolve dried sample in 100 µL of 0.1% TFA and inject onto an HPLC column of PGC.
3. Run a gradient of 0.1% TFA in water (buffer A) and 0.1% TFA in acetonitrile (buffer B) at a flow rate of 1 mL/min with 0–60% buffer B for 60 min, 60% buffer B for 10 min, and 60–0% buffer B for 2 min. Collect 70 fractions of 1 mL and then lyophilize each faction.
4. To assay the amount of hexose present, redissolve each lyophilized fraction by addition of 60 µL of water.

5. Pipet 10 µL of each fraction into a microtiter plate along with a range of concentrations of a hexose standard (Gal or Man, usually 1–10 µg). To each well, add 50 µL of 2% aqueous phenol, mix thoroughly, and leave for 5 min. Add 200 µL of concentrated sulfuric acid to each well and mix prior reading on a benchmark microplate reader at 490 nm (*see* **Note 4**).

3.3. Coupling of Oligosaccharides to PolyL-Lysine (PLL) to Reintroduce Multivalency (see Note 5)

1. To each oligosaccharide fraction recovered from RP-HPLC on PGC (assuming 20 µg of oligosaccharide in each tube), add 50 µL of 0.092 *M* sodium phosphate, pH 7.4, containing 32 µg/mL of PLL (200 pmol in 50 µL) and incubate at 37°C for 2 h.
2. After incubation, add to each fraction 60 nmol of $NaCNBH_3$ (4µg in 5 µL of water) and incubate for 16 h.
3. Prior to lectin overlay analysis, dilute samples 1:2 in phosphate-buffered saline (PBS), and spot 2 × 1 µL of each sample, 1 cm apart, onto a nonmodified NCM (0.2-µm pore size) (*see* **Note 6**).

3.4. Modification and Activation of NCM

3.4.1. Modification of NCM

1. To modify the NCM (0.2-µm pore size) for adherence of peptides and glycopeptides, incubate the membrane with 1:2 divinyl sulfone in dimethylformamide dissolved for 1 h in 0.5 *M* $NaHCO_3$–Na_2CO_3, pH 10.0.
2. Wash the membrane with distilled water and then incubate in 1% aqueous ethylenediamine for 30 min, followed by a wash with water.
3. Incubate the membrane in 1% glutaraldehyde in 0.5 *M* $NaHCO_3$–Na_2CO_3 buffer, pH 10.0, for 15 min.
4. Wash the membrane with water and air dry. The membrane is ready for use for post-HPLC tryptic-digest fractions.

3.4.2. Activation of NCM for Glycoprotein Adhesion

1. To hydrophilically activate NCM without modification (0.45-µm pore size), incubate the membrane in 100% methanol for 5 min.
2. Wash the membrane in distilled water for 1 min and 50 m*M* Tris–0.15 *M* NaCl (TBS) buffer for an additional 1 min and then air dry. The membrane is now ready for loading intact glycoproteins samples.

3.5. Spotting of Samples on the NCM

1. Spot (1 µL) intact non-protease-treated glycoproteins (total protein concentration ranging from 2 µg to 10 ng) or HPLC fractions containing peptides and glycopeptides from the tryptic digests 1 cm apart either onto the activated or modified NCM, respectively (*see* **Notes 7** and **8**).
2. Allow samples to air dry for at least 10 min before identification of glycans (*see* **Note 6**).

3.6. Dot-Blot Lectin Overlay Analysis

3.6.1 Digoxigenin-Labeled Lectins

1. Block the surface of the activated membrane by incubating in the blocking solution (supplied with the kit) for 30 min with gentle shaking (*see* **Notes 9–11**).
2. Wash the membrane twice (5 min each) with at least 50 mL of TBS, pH 7.5.
3. Incubate with the respective lectin solutions: *Datura stramonium* agglutinin (DSA), *Galanthus nivalis* agglutinin (GNA), *Maackia amurenisis* agglutinin (MAA), and *Sambucus nigra* agglutinin (SNA) (an aliquot of the digoxigenin-labeled lectin in 50 m*M* Tris containing 1 m*M* $MgCl_2$ and 1 m*M* $CaCl_2$) for 1 h (*see* **Note 12**).
4. After incubation, wash the membrane 3× with TBS (10 min each).
5. Incubate with anti-digoxigenin (polyclonal sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase) (supplied with the kit) in TBS for 1 h.
6. After washing extensively in TBS, develop the membranes using a freshly prepared staining solution of 4-nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphate (NBT/X-phosphate) (supplied with the kit) in buffer (0.1 *M* Tris-HCl, 0.05 *M* $MgCl_2$, 0.1 *M* NaCl, pH 9.5) (*see* **Notes 13** and **14**).
7. Stop the reaction by washing the membranes extensively (at least 20 changes) in distilled water and leave to air dry (*see* **Note 15**).
8. Scan the membrane using a Hewlett-Packard flat bed scanner (*see* **Notes 16** and **17**).

3.6.2. Biotinylated Lectins

1. Wash the activated or modified membrane twice (5 min each wash), in PBS containing 0.1% Tween 20 (PBS-T) (*see* **Notes 9** and **10**).
2. After washing, block the membrane in PBS-T containing 5% Bovine serum albumin (PBS–T-BSA) for 1 h and wash for a further 4× (5 min each) with at least 50 mL of PBS-T.
3. Dilute in 5 µg/mL of PBS–T-BSA the following biotinylated lectins: *Aleuria aurantia* (AAL), *Lotus tetragononolobus* (LTL), *Lycopersicon esculentum* (LEL), *Maackia amurensis* (MAL-1), *Maclura pomifera* (MPL), soybean agglutinin (SBA), *Sophora japonica* (SJA), *Ricinus communis* agglutinin (RCA-1), *Ulex europeus* II (UEA-II), and wheat germ agglutinin (WGA). Apply respective lectin solutions (50 mL to membranes and incubate for 2 h (*see* **Note 9**).
4. Wash the membranes 4× (5 min each) with at least 50 mL of PBS-T.
5. Incubate in ExtraAvidin peroxidase conjugate, diluted 1:500 in PBS–T-BSA, for 45 min.
6. Wash the membranes 3× in PBS-T and twice in PBS and then incubate in developing solution (60 mg of 4-chloro-1 naphthol dissolved in 20 mL of cold methanol, 80 mL of cold PBS, and 60 mL of 6% hydrogen peroxide) until spots appear on the membrane.
7. Stop the reaction by washing the membranes extensively (at least 20 changes) in distilled water and leave to air dry (*see* **Note 15**).
8. Scan the membrane using a Hewlett-Packard flat bed scanner (*see* **Notes 17** and **18**).

4. Notes

1. For safety, the evaporator is vented to a fume cupboard and kept on overnight after the sample had been dried.
2. To identify and study the site occupancy of individual glycans of glycoproteins, the protein is first cleaved with proteases. The relative hydrophobicities ***(7, 8)*** of the trypsin digest are calculated to predict the elution of the HPLC column as shown for disulfide-bonded Thy-1 (**Table 1**). We have shown earlier that in HPLC of peptides and glycopeptides using a PGC column ***(5)***, the main determinator of retention is peptide and not oligosaccharide; thus, glycopeptides will elute in the same relative order as their peptides. The predicted hydrophobicities of the peptides containing each *N*-glycosylation site had large differences in value (**Table 1**) as mirrored in the time of elution between the glycopeptide HPLC fractions.
3. As an example for Thy-1, of the 35 fractions collected from the HPLC purification, the lectin-DSA (specific for Galβ1–4GlcNAc linkages present in complex and hybrid chains) bound well to fractions 13 and 14 and not as well to fractions 2 and 3. Staining with the lectin-GNA (specific for nonreducing terminal Man present in high mannose and hybrid chains) showed binding to fraction 10 and fractions 8, 9, and 11. This suggested that the least hydrophobic glycopeptide containing site $_{98}$NKT (**Table 1**) had complex chains (factions 2 and 3), the glycopeptide containing $_{23}$NKT has high mannose chains (fractions 8–10), and the most hydrophobic glycopeptide containing $_{74}$NFT had complex chains (fractions 13 and 14). There was some evidence (*not shown*) for staining of fractions 12–14 with GNA as well as DSA, suggesting that hybrid chains could also be present at this site. The GPI anchor glycan attached to the C-terminal tryptic peptideμs) was not expected to interact well with either of the lectins, but may possibly have added to the binding of GNA in the fractions 8–10 due to the Manα1–2Man shown near the protein.
4. The hexose assay is used to quantitate the amount of material after coupling to PLL (*see* **Subheading 3.3**) an dialysis during optimization of the reaction. Do not overfill wells during the hexose assay, as concentrated sulfuric acid will severely damage the microtiter plate reader if spilled.
5. To screen for lectin and antigenic activity to multiple oligosaccharides released from mucin glycoproteins by hydrazinolysis, the oligosaccharides are coupled to PLL, which provides a multivalent molecule to which dot blots adhere throughout washing procedures required for lectin overlay. This is a convenient “one-pot” reaction before purification and detailed quantitative studies of active oligosaccharides can be done.
6. Take care to allow the samples to air dry completely before the second application on the NCM.

Table 1
Theoretical Tryptic Peptides of Thy-1 in Order of Increasing Hydrophobic Character

HPLC[a]	Residues	Sequences[b]	
−15.4	79–99	K <DEGDYMCELRVSGQNPTSSNK> T	Fx 2–3
−12.9	89–99	R <VSGQNPTSSNK> T	complex
−7.9	38–49	R <EK> K	chain on
−4.7	89–105	R <VSGQNPTSSNKTINVIR> D	N^{98}KT
−4.2	38–39	R <EK> K	
−2.6	111–111	K <C>	
−1.1	57–58	R <SR> V	
−0.8	40–41	K <KK> H	
−0.0	106–107	R <DK> L	
−2.7	1–2	<QR> V	
2.9	40–40	K <K> K	
2.9	41–41	K <K> H	
4.1	79–85	K <DEGDYMCELR> V	
8.2	100–107	K <TINVIRDK> L	
10.9	17–20	R <LDCR> H	
14.8	100–105	K <TINVIR> D	
17.2	108–111	K <LVKC>	Fx 8–9
19.8	106–110	R <DKLVK> C	Possible GPI
24.8	21–39	R <HENNTNLPIQHEFSLTREK> K	Anchor at
25.3	57–65	R <SRVNLFSDR> F	VKC- or high
26.4	108–110	K <LVK> C	Man at N^{23} NT
28.7	66–68	R <FIK> V	
33.0	59–65	R <VNLFSDR> F	
35.6	21–37	R <HENNTNLPIQHEFSLTR> E	Fx 10
37.1	42–58	K <HVLSGTLGFPEHTYRSR> V	High Man
39.9	17–37	R <LDCRHENNTNLPIQHEFSLTR> E	at N^{23} NT
41.1	41–56	K <KHVLSGTLGVPEHTYR> S	
44.8	42–56	K <HVLSGTLGVPEHTYR> S	
55.1	59–68	R <VNLFSDRFIK> V	
55.8	1–16	<QRVISLTACLVNQNLR> L	
59.7	3–16	R <VISLTACLVNQNLR> L	
64.0	3–26	R <VISLTACLVNQNLRLDCR> H	
67.1	69–88	K <VLTLANFTTKDEGDYMCELR> V	Fx 13–14
69.6	69–78	K <VLTLANFTTK> D	Complex and hybrid at N^{74} FT
91.7	66–78	R <FIKVLTLANFTTK> D	

[a] Relative hydrophobicity index on C18 HPLC.

[b] < > denotes the possible alternative tryptic cleavage sites.

7. To avoid unnecessary cross-contamination between samples, a 96-well dot-blotter (Bio-Rad) was used instead of spotting the samples directly onto the membrane. Prior to activation or modification of the NCM, cut the membrane to size for placing onto the dot blotter cassette. Once the membrane is in a fixed position, apply respective samples as indicated in **Subheading 3.5**. After loading samples remove membrane from dot blotter and leave to air dry. Once dried, mark the membrane with a pencil to indicate the order of the samples. Also, draw a grid on paper to indicate the location of samples.
8. When applying respective samples onto the membrane, always use fresh Gilson tips for each sample to avoid contamination.
9. All incubations in the lectin overlay analysis are carried out on a rocking table at room temperature.
10. During the lectin overlay analysis, use gloves and avoid direct contact of the membrane with skin. Handle the membrane carefully with a flat-ended twiser to avoid damage.
11. For a clearer background, the blocking solution can be incubated with the membrane for overnight at 4°C. After, incubate the membrane with the blocking solution for a further 30 min at room temperature prior to washing.
12. For convenience, use large Petri dishes to incubate each membrane with respective lectin solutions. Make sure that the lectin solution always covers the membrane surface during incubation.
13. In contrast to the manufacturer's instructions the staining reaction can be extended until significant staining could be observed (1–18 h).
14. Standard glycopeptides should also spotted onto the membrane to monitor the progress of staining.
15. To protect the membrane from dust and damage, place a clear plastic film over the membrane and store in the dark.
16. **Table 2** presents a summary of results of a series of whole non-protease-treated glycoproteins (at various concentrations) analyzed for lectin specificity and sensitivity by dot-blot lectin overlay. The results from this assay

Table 2
The Sensitivity Levels of Commercial Lectins on Glycoproteins by Lectin Overlay Analysis

Lectin	Fetuin	Asialylated fetuin	Bovine serum albumin	Chicken ovalbumin	RNase B
MAA	10 ng	1 μg	NB	NB	NB
SNA	0.1 μg	NB	NB	NB	NB
DSA	0.1 μg	0.1 μg	NB	NB	NB
GNA	1 μg	1 μg	2 μg	1 μg	NB

NB, No binding of lectin detected to sample.

clearly show that three lectins (MAA, SNA, and DSA) recognize Bovine fetuin (BF) at high sensitivity protein levels ranging from 10 ng to 0.1 µg. Asialylated BF (ABF) were detected by lectins MAA and DSA but not SNA. As expected, all three lectins showed no binding to Bovine serum albumin (BSA), which is not glycosylated. According to the lectin specificity as published by the manufacturer, GNA should stain positive for RNase B and ovalbumin but not the other glycoproteins/proteins; however, negative results with RNase B were shown even after repeated tests such as using increased concentrations of RNase B (up to 20 µg). The sugar of RNase B was analyzed by hexose assay and shown to contain the expected 3%; thus the specificity of GNA is not as published, and the lectin can distinguish between ovalbumin and RNase B glycosylation.

17. **Table 3** shows the binding preferences of the lectins used. From the results obtained, the sensitivity of lectins can detect protein concentrations down to 10 ng. The choice between the commercial digoxigenin-labeled and biotinylated lectins used relate to their recognition of a range of defined and limited sugar structures depending on the samples tested. One drawback to the procedure is that because a number of structurally distinct oligosaccharides interact identically with certain lectins, this suggests that the structural heterogeneity frequently encountered with glycoproteins may not always be reflected in the interactions of these moieties with lectins.
18. For pigeon intestinal mucin ***(2)*** 8 of the 10 lectins (AAL, LEL, LTL, MAL-1, MPL, RCA-1, SBA, SJA, UEA-II, and WGA) reacted specifically with a different spectrum of PLL-oligosaccharide conjugates. LTL stained the entire blot, suggesting that this lectin interacted with the nitrocellulose. SJA reacted with all of the fractions and controls, indicative of nonspecific interaction with PLL itself. For the other lectins there was a wide range of activities with the various HPLC fractions. Difference in staining patterns between the lectins demonstrated that oligosaccharides of varying structure have been released from pigeon mucin by hydrazinolysis and have successfully been coupled to PLL, thus allowing binding to the NCM. Optimization of conditions at the macro-level included both neutral and sialylated oligosaccharides, with the chosen conditions giving maximum yield of both. The principle of this assay is derived essentially from enzyme-linked immunosorbent assay (ELISA) and commercial kits are available, where lectins are already linked to antibodies or other methods for detection.

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Table 3
Binding Preferences of Digoxigenin-Labeled and Biotinylated Lectins

Lectin	Specificity	**References**
Digoxigenin-labeled Lectins		
Datura stramonium agglutinin	Galβ1–4GlcNAc in complex and hybrid *N*-glycans, in *O*-glycans and GlcNAc in *O*-glycans	***9***
Galanthus nivalis agglutinin	Terminal Man, α1–3, α1–6, or α1–2 linked to Man; suitable for identifying "high Man" *N*-glycan chains or *O*-glycosidically linked Man in yeast glycoproteins	***10***
Maackia amurensis agglutinin	Sialic linked α2–3 to galactose; suitable for indentifying complex, sialylated carbohydrate chains and type of sialic acid linkage	***11***
Sambucus nigra agglutinin	Sialic acid linked α2–6 to galactose; suitable for identifying complex, sialylated *N*-glycan chains in combination with lectin MAA	***12***
Biotinylated lectins		
Aleuria aurantia lectin	Fucose linked α1–6 to GlcNAc or to fucose linked α1–3 to *N*-acetyllactosamine	***13***
Lotus tetragonolobus lectin	α-Linked L-fucose containing oligosaccharides	***14***
Lycopersicon esculentum (tomato) lectin	Trimers and tetramers of GlcNAc	***15***
Maclura pomifera lectin	α-Linked GalNAc	***16***
Ricinus communis agglutinin I	Terminal galactose and GalNAc	***17***
Sophora japonica agglutinin	Terminal GalNAc and galactose residues, with preferential binding to β-anomers	***18***
Soybean agglutinin	Terminal α- or β-linked GalNAc, and to a lesser extent, galactose residues	***19***
Ulex europaeus agglutinin II	2′ Flucosyllactose (fucosyl α1–2 galactosyl β1–4 glucose)	***14, 20***
Wheat germ agglutinin	Terminal GlcNAc or chitobiose and sialic acid	***21***

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Polyacrylamide Gel Electrophoresis of Fluorophore-Labeled Carbohydrates from Glycoproteins

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1. Introduction

Prior to 1980, most methods for analysis of glycoprotein carbohydrates utilized column, thin-layer, and paper chromatography, gas chromatography, mass spectroscopy, and rarely nuclear magnetic resonance spectroscopy. These methods required relatively large amounts of materials (micromoles), specialized training and experience, and in some cases, significant capital equipment outlays. Because of these restrictions, convenient carbohydrate analysis on small samples was not available to most biologists. Recently, improvements in chromatographic methods, labeling methods for carbohydrates, carbohydrate-specific enzymes, and higher resolution electrophoresis methods have allowed carbohydrate analysis to be done on nanomolar amounts of material. Because of these improvements, today's biologist now has an improved ability to evaluate the role of carbohydrates in their research and development work.

Polyacrylamide gel electrophoresis (PAGE) of fluorophore-labeled carbohydrates has also been referred to as fluorophore-assisted carbohydrate electrophoresis, or FACE®. The technique was first developed in England by Williams and Jackson ***(1–4)*** and utilizes reductive amination of carbohydrates by low molecular weight, negatively charged or neutral fluorophores and electrophoresis on 20–40% polyacrylamide slab gels. This method permits separation of charged or uncharged sugars or oligosaccharides with high resolution and can detect single hydroxyl anomeric differences between mono- and oligosaccharides of sugars with otherwise identical molecular weight, charge, and sequence. The separation of sugars by electrophoresis is largely empirical, and it is not always possible to predict relative mobilities of structures. For the most part the success

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of this approach has been based on experimental observations and the use of highly specific reagents, enzymes, and standards.

What follows in this chapter are descriptions of the materials and methods required to perform two of the most common manipulations of oligosaccharides used in biologic research today. The method with some modification is useful for analysis of a variety of carbohydrates including reducing and nonreducing sugars, substituted and unsubstituted monosaccharides, and oligosaccharides from glycoproteins, proteoglycans, glycolipids, and polysaccharides. In this chapter only profiling of *N*-linked and *O*-linked glycoprotein oligosaccharides is discussed. The method is also useful for a variety of other manipulations including structure–function studies, preparative work, and synthesis of oligosaccharides that are not discussed in detail here. Other reviews of this technique have been published previously ***(5,6)*** and a number of research studies have been performed using this method ***(7–31)***.

2. Materials

2.1. N-linked Oligosaccharide Analysis

For profiling of Asn-linked oligosaccharides released by peptide *N*-glycosidase F.

1. *N*-linked Gels: 10 × 10 cm low fluorescence glass plates with 0.5 mm spacers and 8-well combs filled with 20% T acrylamide:bis in 0.448 *M* Tris-acetate buffer pH 7.0 and containing a 5-mm stack of 10% polyacrylamide in the same buffer (gels should be made fresh or obtained precast from a commercial source).
2. Releasing enzyme: peptide *N*-glycosidase F (from commercial sources).
3. Running buffer: 50 m*M* Tris tricine buffer (pH 8.2).
4. Enzyme buffer: 100 m*M* sodium phosphate buffer (pH 7.5).
5. Labeling dye: 1 *M* 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) in 15% acetic acid (reagent is stable for 2 wk at–70°C in the dark).
6. Reducing agent: 1 *M* sodium cyanoborohydride ($NaBH_3CN$) in dimethyl sulfoxide (DMSO) (reagent is stable for 2 wk at–70°C).
7. Sample loading solution: 25% glycerol with thorin 1 dye (store at 4°C).
8. Tracking dye: Mixture of Thorin 1, Bromphenol blue, Direct red 75, and Xylene cyanole in water.
9. Electrophoresis gel box (with 2-sided cooling).
10. Deionized or distilled water.
11. Assorted pipeting devices including a 0–10 µL positive displacement pipet (e.g., Hamilton syringe).
12. Centrifugal vacuum evaporator.
13. Oven or water bath at 45°C and 37°C.
14. Sodium dodecyl sulfate (SDS), 5%.
15. β-Mercaptoethanol (βME).
16. 7.5% nonidet P-40 (NP-40).
17. 100% Cold ethanol (undenatured).

18. Microcentrifuge tubes (1.5 mL).
19. Microcentrifuge.
20. Chicken trypsin inhibitor control (optional).
21. Maltotetraose or partially hydrolyzed starch standard (optional).

2.2. O-linked Glycoprotein Oligosaccharide Analysis

For profiling of Ser/Thr-linked oligosaccharides released by hydrazine.

1. *O*-linked gels: 10 × 10 cm low fluorescence glass plates with 0.5-mm spacers and eight-well combs filled with 35% T acrylamide:bis in 0.448 *M* Tris-acetate buffer and containing a 5-mm stack of 16% polyacrylamide in the same buffer (gels should be made fresh or obtained as precast gels from a commercial source).
2. Gel running buffer: 50 m*M* Tris-glycine (pH 8.2).
3. *O*-linked cleavage reagent: anhydrous hydrazine, 1 mL amp. **Hydrazine is toxic and flammable; discard ampoule and residual contents after using once; dispose of safely according to your institution's regulations.**
4. Re-*N*-acetylation reagent: acetic anhydride.
5. Re-*N*-acetylation buffer: 0.2 *M* ammonium carbonate, pH 9.4.
6. Desalting resin: Dowex AG50X8.
7. Tracking dye: Mixture of Thorin 1, Bromphenol blue, Direct red 75, and Xylene cyanole in water.
8. Sample loading solution: 25% glycerol with Direct red 75 (store at 4°C).
9. Labeling dye: 1 *M* 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) in 15% acetic acid (reagent is stable for 2 wk if stored in the dark at –70°C).
10. Reducing agent: 1 *M* sodium cyanoborohydride ($NaBH_3CN$) in DMSO (reagent is stable for 2 wk at –70°C).
11. Distilled-deionized water.
12. Microcentrifuge tubes (2-mL, glass-lined).
13. Assorted pipeting devices including a 0–100 µL capillary positive displacement pipet.
14. Centrifugal vacuum evaporator.
15. Oven or water bath set at 37°C.
16. Sand filled heat block set at 60°C.
17. Vacuum dessicator.
18. Microfuge.
19. Phosphorus pentoxide (P_2O_5).
20. Bovine submaxillary mucin control (optional).
21. Maltotetraose or partially hydrolyzed starch standard (optional).

3. Methods

3.1. Principle

Using fluorescent PAGE, individual oligosaccharides can be quantified to obtain molar ratios, to obtain degree of glycosylation, and to detect changes in the extent or nature of glycosylation. Oligosaccharide profiling involves four steps:

1. Release of the oligosaccharides from the glycoprotein enzymatically or chemically;
2. Labeling of the mixture of released oligosaccharides with a fluorescent tag;
3. Separation of the fluorophore-labeled oligosaccharides by PAGE; and
4. Imaging of the gel either on a UV lightbox to obtain qualitative band information or using a commercial imaging system to determine the amount of oligosaccharide present in each band and the relative mobility of the bands.

Once separated on the gel, individual oligosaccharide bands can also be purified for further study.

3.2. Preparation of Glycoprotein

1. Isolate the glycoprotein according to your usual procedures. The sample should be relatively salt-free and contain no extraneous carbohydrates (e.g., sephadex-purified material contains large amounts of glucose) (*see* **Note 1**).
2. If the volume of the glycoprotein solution required is >100 µL, dry the glycoprotein in a 1.5-mL microcentrifuge tube. Generally 50–200 µg of glycoprotein is required for *N*-linked analysis (*see* **Note 2**). For analysis of *O*-linked oligosaccharides 100–500 µg of glycoprotein may be required.
3. For the *N*-linked oligosaccharide control (*see* **Note 3**) remove 100 µg of the chicken trypsin inhibitor in a 45 mL aliquot, and place it in a 1.5 mL microfuge tube. Proceed with the enzymatic digestion as described below. For the *O*-linked oligosaccharide control remove 50 µL (100 µg) of Bovine submaxillary mucin, and place it in a reaction vial. Proceed with lyophilization, P_2O_5 drying and process along with the sample glycoprotein (*see* **Subheading 3.4.2.**). Store remaining glycoproteins at 4°C for future use.

3.3. Release of Asn-Linked Oligosaccharides with Peptide N-Glycosidase F

1. Add an equal volume of enzyme buffer to the glycoprotein in solution or dissolve the dried glycoprotein in 22.5 µL of water and add 22.5 µL enzyme buffer.
2. SDS is often required to completely denature the glycoprotein prior to enzymatic digestion. To denature the protein add SDS to 0.1% (1.0 µL of 5% SDS to 45 µL reaction) and β-ME to 50 m*M* (1.5 µL of a 1:10 dilution of 14.4 *M* stock β-ME to 45 µL reaction). Boil for 5 min (*see* **Note 4**), cool to room temperature and add NP-40 to 0.75% (5 µL of 7.5% NP-40 into 45 µL). Mix with finger flicks.
3. Add 2.0 µL (5 U of peptide-*N*-glycosidase F or as specified by the manufacturer) of enzyme to the glycoprotein sample. Mix with finger flicks and centrifuge for 5 s. Store remaining enzyme at 4°C.
4. Incubate sample for 2 h at 37°C.
5. Precipitate protein by adding 3 vol of cold 100% ethanol.
   Keep samples on ice for 10 min. Spin samples in microcentrifuge for 5 min to pellet protein.
6. Remove the supernatant and transfer to a clean 1.5-mL microcentrifuge tube.
   IMPORTANT! Do not discard the supernatant. It contains the released carbohydrates!

7. If a large amount of protein was digested (>250 µg) 5–10% of the released oligosaccharides may remain in the pellet. The recovery of these oligosaccharides can be accomplished by drying the pellet completely in a centrifugal vacuum evaporator or lyophilizer. Add 50 µL H_2O to resuspend, then 150 µL 100% cold ethanol and precipitate on ice. Centrifuge and combine the supernatants.
8. Dry the supernatants in a centrifugal vacuum evaporator or lyophilize to a translucent pellet. At this point samples may be stored at −20°C or proceed with the fluorophore labeling procedure described in **Subheading 3.4.2**.

3.4. O-Linked Oligosaccharide Release Using Hydrazine

3.4.1. Isolation of Glycoprotein

1. Isolate glycoprotein according to your usual procedures. The purified glycoprotein should be prepared in a non-Tris buffer containing a minimum amount of salt. The presence of nonvolatile salts may cause the breakdown of the oligosaccharides during hydrazinolysis. If the glycoprotein is in a buffer containing salt it is recommended that the sample be dialyzed against distilled water to remove salts prior to chemical digestion.

3.4.2. Hydrazinolysis

1. Dry 100–500 µg of glycoprotein in a glass-lined reaction vial, using a centrifugal vacuum evaporator or lyophilizer. The actual amount of glycoprotein required will depend on the size of the protein and the extent of glycosylation (*see* **Note 2**).
2. The sample must be completely dry before hydrazinolysis. Following lyophilization, dry the sample overnight under vacuum in the presence of P_2O_5 to remove all traces of H_2O. Place samples in a dessicator flask with a beaker containing a small amount of P_2O_5. Attach the dessicator directly to the pump without a cold-trap—any water remaining in the sample will be trapped by the P_2O_5.
3. Open a fresh ampoule of anhydrous hydrazine *O*-linked cleavage reagent. Add 50 µL of hydrazine to the dried sample using a glass transfer pipet, or a positive displacement capillary pipet (metal or plastic should not be used). Resuspend the dried sample. Overlay the sample with dry nitrogen and cap tightly. **Hydrazine is very hygroscopic. Discard unused hydrazine according to your hazardous waste regulations. Do not reuse**.
4. Incubate samples for 3 h in a sand bath or dry heat block set at 60°C (do not use a water bath) to release *O*-linked oligosaccharides (higher temperatures may result in the degradation of *O*-linked sugars or in the release of non-*O*-linked sugar chains, such as *N*-linked sugars, from the sample if they are present).
5. Dry samples in vacuum evaporator on low heat setting.

3.4.3. Re-N-Acetylation Procedure

1. Add 30 µL of Re-*N*-acetylation buffer to the dried pellet from **step 5** in **Subheading 3.4.2.**
2. Resuspend by vortexing. Spin 2 s in a microfuge.
3. Add 2 µL of re-*N*-acetylation reagent to the solution. Mix well. Spin 2 s in a microfuge.

4. Incubate tubes on ice for 15 min.
5. Following the 15 min incubation, stop the reaction by adding 60 µL of the desalting resin. Desalting resin is prepared by adding 0.5 g of Dowex AG50X8 to 0.7 mL water. Invert or vortex the resin immediately prior to removing the 60 µL for each tube. Incubate the resin with the sample at room temperature for 5 min mixing by placing the tube on a shaker or by continuously inverting the tube to keep the resin suspended.
6. Briefly centrifuge to pellet the resin, remove the supernatant (save supernatant) and wash the resin 2× with 120 µL of water for 2 min each (save supernatant).
7. Combine the resin supernatants in a 1.5-mL microcentrifuge tube.
8. Dry the supernatants in a vacuum evaporator on low heat setting.

3.5. Labeling Oligosaccharides

3.5.1. Preparation of Samples and Standards

If quantitation of the oligosaccharide bands in the samples is required, then one must compare the intensity of an internal standard (e.g., maltotetraose) band with the intensity of sample bands and it is, therefore, essential that the standard is present on each gel used (*see* **Note 5** for preparation and use of this material).

3.5.2. ANTS Labeling

1. Prepare the labeling dye as 1 *M* ANTS in 15% acetic acid (dye solution can be stored in the dark at –70°C for up to 2 wk).
2. Prepare 1 *M* solution of $NaBH_3CN$ in DMSO and mix well by vortexing until crystals are completely dissolved (this reducing agent can be stored for 2 wk at –70°C).
3. Add 5 µL of labeling dye to each dried oligosaccharide pellet. Mix well until the oligosaccharide pellet is dissolved.
4. Add 5 µL of reducing agent. Mix well by vortexing. Centrifuge 5 s in microcentrifuge.
5. Incubate samples at 45°C for 3 h (temperatures higher than 45°C or times longer than 3 h can destroy or modify carbohydrates, e.g., sialic acids). Greater than 90% of the oligosaccharides are labeled under these conditions. As a convenient alternative samples can be labeled at 37°C (not 45°C) overnight (or approx 16 h). These latter conditions result in labeling of >90% of the oligosaccharides (*see* **Note 6**).
6. After labeling, dry the samples in centrifugal vacuum evaporator for approx 15 min or until the sample reaches a viscous gel stage.

3.6. Electrophoresis

3.6.1. Preparation of a Sample for Electrophoresis

1. Resuspend the dried fluorophore “labeled” oligosaccharide in 5–20 µL H_2O. The actual volume of H_2O used to resuspend the sample will depend on the amount of oligosaccharide present in the sample (start with 10 µL, this will enable the sample to be diluted further if necessary).

2. Remove an aliquot of the sample (generally 1–2 µL) and dilute it with an equal volume of sample loading solution. Load the entire aliquot into one lane of a gel. Best results are obtained by loading 4 µL/lane on a 10 × 10 cm gel with eight lanes.

3.6.2. Electrophoresis

1. For *N*-linked analysis chill the running buffer to 4–6°C prior to use. For *N*-linked analysis perform electrophoresis at a buffer temperature of 5–8°C. All *O*-linked gels are run at 15–20°C.
2. For *N*-linked analysis set up electrophoresis with a recirculating chiller and place the electrophoresis tank containing a stir bar on a mechanical stirrer. Connect the gel box cooling chamber to a refrigerating circulator. Turn on the circulator and stirrer and set the coolant temperature to 5°C.
3. For *N*-linked analysis pour the precooled running buffer into the electrophoresis tank up to the appropriate level. The temperature of the buffer should be monitored during the run using a thermometer inserted through the hole in the lid or other method. The temperature will probably increase a few degrees during electrophoresis, but should not exceed 10°C. For *O*-linked gels the temperature should not exceed 23°C.
4. Determine the number of gels required for the samples prepared. Each gel should contain eight lanes. The outside lanes should be used for the tracking dye and glucose polymer standard leaving the six inner lanes for samples and quantitation standard (maltotetraose).
5. Gently remove the comb(s) from the gel(s). To avoid distorting the wells, gently wiggle each comb to free the teeth from the gel, then lift up slowly until the comb is released.
6. Place the gel cassette(s), one on each side of the center core unit of the gel apparatus with the short glass plate against the gasket. Be sure the cassette is centered and that the cassette is resting on the “feet” at the bottom of the apparatus. If only one gel is being run place the buffer dam on the other side.
7. It is essential that the wells of the gel are thoroughly rinsed out with the running buffer from the upper buffer reservoir prior to sample loading. This is best accomplished by using a syringe with a blunt needle (a Pasteur pipet is not recommended because of the possibility of breakage into the wells).
8. With the core unit containing the gels placed securely on the bench, load samples into the wells by underlaying the upper buffer. Use flat sequencing pipet tips to load by delivering the sample to the bottom of each well. Optimal resolution will be achieved by using 4 µL of sample per lane.

   Note: For the most reliable quantitation of oligosaccharide bands the use of a positive displacement pipet (e.g., Hamilton syringe) is recommended.
9. Load 4 µL of the standard in a lane when prepared as described in **Note 5**.
10. Load 2 µL of tracking dye in a lane directly from the vial.
11. Load 4 µL of each labeled oligosaccharide sample in a lane. Samples should be diluted 1:1 in the sample loading solution (*see* **Note 7**).

12. To prevent possible lane distortions as a result of different loading volumes it is recommended that **4** µL of Sample Loading Solution be loaded in any unused lanes. Best results are obtained when the same volume of sample is added to each lane.
13. Place the core unit containing the loaded gels into the electrophoresis tank and connect the power cords to the electrophoresis tank then connect the power supply.
14. Place the thermometer into the lower buffer chamber through the hole in the lid. For *N*-linked analysis the initial temperature of the lower buffer must be 5–8°C; for *O*-linked analysis 15–20°C is optimal.
15. Turn on the power supply and select the proper current. Gels should be run at a constant current of 15 mA/gel (30 mA for 2 gels; 15 mA for 1 gel and 1 buffer dam). Limits on the power supply should be set for 1000V and 60W. These run conditions will result in voltages of 100–400V at the beginning of the run and may approach 800–1000V at the end of the run. If the initial voltage is significantly different check to be sure that the leads are connected properly and that the buffers are at the recommended levels (*see* **Notes 8** and **9**).
16. Most *N*-linked oligosaccharides fall in the Glucose_4–Glucose_{12} range (also referred to as G4–G12 or DP4–DP12), so the time of electrophoresis should be adjusted to optimize the separation of this region of the gel. Most *O*-linked oligosaccharides run in the G1–G6 range (DP1–DP6).
17. Monitor the electrophoresis by following the migration of the fast moving thorin dye (orange band). Generally, electrophoresis of *N*-linked oligosaccharides is complete when the orange dye just exits the bottom of the gel in approx 1 to 1¼ h. For the *O*-linked oligosaccharides the gel run is complete when the orange dye is 1 cm above the bottom of the gel. In a darkened room, the migration of the labeled oligosaccharides can be monitored directly during electrophoresis by turning off the power supply, removing the leads and the gel box cover and holding a hand-held UV light over the gels. The run can be continued by repositioning the gel in the electrophoresis box and reconnecting the power supply as described in **step 15**. The amount of time the current is off should be as short as possible (<5 min) to minimize diffusion of the oligosaccharides in the gel.
18. When the electrophoresis is complete, turn off the power supply. Disconnect the power cords from the power supply and the electrophoresis tank. Turn off the refrigerated cooler and discard the buffer (*see* **Note 10**).

3.7. Gel Imaging

CAUTION: UV protective eyeware or faceshield should be worn. Avoid prolonged exposure to UV light.

1. Allow UV lightbox to "warm-up" for at least 2 min in order to get maximum intensity output. The lightbox must be long-wave UV and have a peak output at approx 360 nm; this is *not* the type of box typically used for ethidium bromide-stained DNA gels.
2. Remove the tape from the gel cassette, which may be fluorescent, and clean the surfaces of the cassette if it is required to image the gel within the cassette.

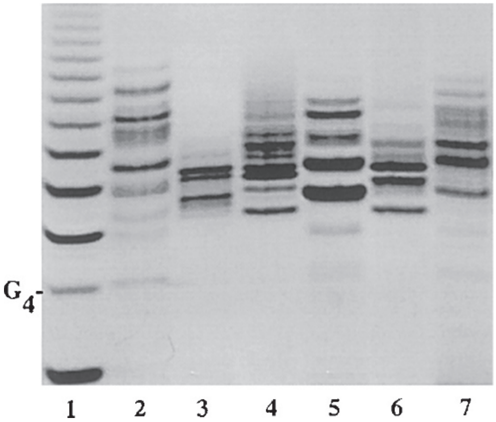

Fig. 1. Profiles of *N*-linked oligosaccharides from several glycoproteins. ANTS-labeled oligosaccharides released by peptide *N*-glycosidase (PNGase F) from six different glycoproteins are shown. *Lane 1* contains an oligosaccharide ladder standard of partially hydrolyzed wheat starch with G4 representing glucotetraose; *lane 2*, chicken trypsin inhibitor; *lane 3*, bovine fetuin; *lane 4*, human α-acid glycoprotein; *lane 5*, bovine ribonuclease B; *lane 6*, human chorionic gonadotropin (hCG); and *lane 7*, chicken ovalbumin. The profiles show a wide variety of different glycosylation patterns, indicating the minimum number (some oligosaccharides will comigrate) of different types of oligosaccharide present and their relative quantities. The images presented in this review were obtained by imaging the gels following electrophoresis using a CCD (charge-coupled-device)-based imaging system (Glyko).

The cassette glass must be of special low-fluorescence type to obtain an image of a gel within the cassette. If the glass is not of a low fluorescence type and if the type of gel cassette being used permits disassembly, remove the gel completely from the cassette and place it on the UV lightbox (*see* **Notes 11** and **12**).

3. An electronic image of the *N*-linked oligosaccharides from several glycoproteins is shown in **Fig. 1**; an electronic image of the *O*-linked oligosaccharides from several different glycoproteins is shown in **Fig. 2**. In each image you can also see the position of maltotetraose and a ladder of maltooligosaccharides from partially hydrolyzed wheat starch (*see* **Notes 9, 10, 13–16** for problems with bands).

3.8. Gel Handling

1. If the gel is no longer needed it should be properly discarded.
2. Following imaging of the oligosaccharide gels, the glass plates can be separated and the gels dried on a flat bed gel drier between sheets of Teflon™ membrane at 80°C for 1 h. After the gel is dry, carefully peel the Teflon sheets away from the gel. Gels dried in this manner can be stored indefinitely in a dark dry location and can reimaged at any time with minimal bleaching.

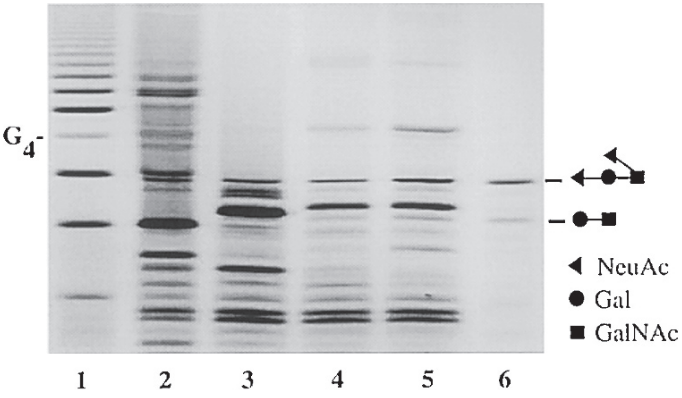

Fig. 2. Profiles of *O*-linked oligosaccharides from several glycoproteins. ANTS-labeled Ser/Thr-lined oligosaccharides released using hydrazine at 60°C from four different glycoproteins are shown. *Lane 1* contains an oligosaccharide ladder standard of partially hydrolyzed wheat starch with G4 representing glucotetraose; *lane 2*, porcine stomach mucin, Type II; *lane 3*, bovine submaxillary mucin, Type I; *lane 4*, bovine fetuin; and *lane 5*, human chorionic gonadotropin (hCG). *Lane 6* contains the NeuAc(α2–3)Gal(β1–3)[NeuAc(α2–6)]GalNAc and Gal(β1–3)GalNAc standards. The images presented in this review were obtained by imaging the gels following electrophoresis using a CCD-based imaging system (Glyko).

3. Following imaging, the gel cassette can be placed back in the electrophoresis apparatus and the run continued in order to improve the resolution of the oligosaccharide bands. In this case, the *upper* buffer should be saved and reused until the run is finally terminated. Note that diffusion of carbohydrate bands and subsequent poor resolution will occur if the time between electrophoresis and imaging exceeds 15 min.

4. Notes

1. The glycoprotein sample should ideally first be dialyzed against distilled water and stored lyophilized in a 1.5 mL microfuge tube. If the sample needs to be in a buffered solution, one can place the sample in 50 m*M* sodium phosphate buffer, pH 7.5, at a final concentration of at least 100 µg/50 µL or 2 mg/mL. Best results are obtained if the total salt concentration of the solution is <100 m*M*. The use of a Tris-based buffer is not recommended. If a detergent is required, the sample may be suspended in up to 0.1% SDS, 0.75% NP-40, or 1% n-octyl β-D-glucopyranoside. If desired, the sample may also contain 0.05% sodium azide.
2. We recommend that you use at least 250 µg of glycoprotein for analysis. The actual amount of glycoprotein required for profiling will depend on the size of the protein, the amount of glycosylation, and the degree of oligosac-

charide heterogeneity. In general, the amount of glycoprotein required increases with the size of the protein or the degree of heterogeneity and decreases with the percent of glycosylation. As a general guideline, one would start with approx 50–100 µg to profile the *N*-linked oligosaccharides of a 60 kDa glycoprotein that contains 10–20% carbohydrate by weight. For *O*-linked oligosaccharide analysis we suggest 100–500 µg of starting glycoprotein. This amount would normally provide sufficient material for several electrophoretic runs. For isolation of individual oligosaccharides, and carrying out sequencing, additional material will be required.

3. The control for *N*-linked profiling consists of trypsin inhibitor that is used as a control for enzyme digestion and fluorophore labeling. This control should be included in the analysis for the following reasons:
   a. If this control is used for the first time it will help the user to become familiar with the procedures;
   b. If the profile obtained looks appropriate then this assures the user that things are working properly; and
   c. In an unknown sample that may not contain *N*-linked oligosaccharides, the user can be certain that the reagents are good and that the release and labeling procedures were performed properly.

   Similarly a control for *O*-linked profiling such as Bovine submaxillary mucin should be used.
4. Some proteins will precipitate when boiled, i.e., immunoglobulins. The following procedure should be used if your protein precipitates: Add SDS/ -ME at the recommended concentration and incubate for 5 min at room temperature, add NP-40 according to directions, and then add PNGase F and incubate overnight.
5. The quantitation control consists of maltotetraose (Glucose 4). This control should be prepared so that once prepared 5 µL will contain 200 pmol of maltotetraose (standards prelabeled with ANTS are also available commercially). Accurate quantitation will be achieved when using an electronic imaging system. If using a commercial imaging system, refer to the manual for a detailed description of the quantitation procedures.
6. The stoichiometry of labeling is such that only one molecule of fluorophore is attached to each molecule of oligosaccharide. When labeling 20 nmol or less of total sugar using the reagents and labeling conditions described, the fluorophore labeling efficiency is >95% (5). Labeling more than 20 nmol in each reaction will result in reduced labeling efficiency. When labeling >20 nmol it is recommended that an internal labeling control is included.

7 Sample handling and storage:
   a. Always avoid exposing labeled samples and dyes to light or excess heat;

   b. Labeled samples are stable when stored for 3 mo at –70°C in the dark;
   c. Unused solutions of the dye and reducing agent can be stored for as long as 2 wk at –70°C. Thaw immediately before use.
8. Band distortion in gels caused by vertical streaking or smearing may result if the sample is overloaded. Use a maximum of 1/5 of the volume of the labeling reaction for each lane. The sample may have a high concentration of salt. Remove salts by dialysis, desalting column, and so on, prior to enzymatic digestion. Distorted sample wells in gel may be caused by tearing of wells when the comb was removed. Remove the comb slowly using a gentle back and forth rocking motion and lift vertically. Alternatively, gels may have been in contact with upper buffer too long prior to sample loading. Samples should be loaded within 5 min of placing the gel in the upper buffer tank.
9. Voltage and/or current leaks can result when high voltages are used. If at the beginning of the run the voltage is >400V or readings are unstable, turn the power off before checking the following: possible electrical leak, check for cracks in glass plates. Remove inner core assembly and check for buffer leak between gaskets and cassette plates. If leaks are evident check that the plates are clean and not cracked or chipped, and that they are installed properly.
10. Buffers should not be reused as they have fluorophore contamination after use. Reuse of buffers may result in no bands being visible on the gel owing to “washout” of the fluorophore-labeled oligosaccharides.
11. Accurate quantification is essential for detailed carbohydrate analysis. Although oligosaccharide patterns on PAGE gels can be viewed and photographed on a standard laboratory UV lightbox, it is not reliable for accurate quantification. Images of gels can be recorded using a Polaroid camera. The proper choice of light source, filters, and film must be made. A filter must be fitted to the camera lens that completely covers the glass of the lens (stray UV contacting the lens will cause it to fluoresce and subsequently lower the sensitivity of the film). A suitable filter will have no inherent fluorescence, peak transmission at approx 500 nm and bandwidth of 80 nm FWHM. A medium speed, medium resolution, Polaroid film is recommended. Use Polaroid 53 film for cameras which use single 4 × 5″sheet film; use Polaroid 553 film for cameras that use 8 sheet film cartridges.

    To visualize the carbohydrate banding patterns, the low fluorescent glass cassette containing the gel (or the gel removed from the casette) is placed on a longwave UV lightbox with a peak excitation output at approx 360 nm.

    Photograph the gel using the lowest practical f-stop setting on the lens with the gel filling as much of the frame as possible. E.g., exposures at f5.6

using Polaroid 53 film have ranged from 5–40 s using the equipment specified above. Keep UV exposure of the gel to a minimum to prevent bleaching.

Develop the film according to the manufacturer's instructions.

12. For electronic archiving and quantitation, several types of imaging systems are available. To give best results these systems must have an illumination source with an excitation wavelength of 365 nm and a 520 nm emission filter placed in the light path between the gel and the image capturing device. The use of an internal standard in the gels is also required for quantitation.

    Following electrophoresis, the gel is inserted into the imager under longwave UV excitation, and an electronic image of the fluorescent carbohydrate banding pattern of the gel is acquired by the imager's CCD as a digital image. The gel image is displayed on a computer screen using the imaging software. The imaging system should allow for detection and quantification of individual carbohydrate bands into the low picomole range of 1.6–300 pmol. In practice, the most useful and accurate range of the imager for band quantification is between 5 and 500 pmol of carbohydrate and this range was used for the experiments described in this chapter.
13. You may have "smile effect" gel distortions at both sides of the gel. This can happen if the gel is not being cooled uniformly. Check that the cooling system is on and working properly. Check the buffer temperature. Check that the power supply is set for the proper current level.
14. You may have band distortions or "fuzzy bands." This can be caused by wells that may have not been rinsed thoroughly with electrophoresis upper buffer prior to loading samples, or the current may have not been set properly, i.e., the current was too high.
15. Incomplete re-N-acetylation may result in little or no labeling of the released oligosaccharides, presumably by the hydrazide interfering with the reductive amination using ANTS. To check the re-N-acetylation reagents you can use glucosamine that will migrate at a DP of approx 2.5 on the gel when N-acetylated and below DPI when unacetylated. You can also use N-acetyl-glucosamine as an internal control at the beginning of the experiment and take it through hydrazinolysis and re-N-acetylation expecting the same migrations as stated.
16. Oligosaccharides are small molecules that can diffuse rapidly in the gel matrix. Band diffusion and resulting broad or "fuzzy" bands can occur if:
    a. Electrophoresis is run too slowly;
    b. Electrophoresis is run at higher than optimal temperatures;
    c. Electrophoresis is stopped and started repeatedly; and
    d. The gel is removed for visualization for longer than 10 min and then re-electrophoresed.

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HPLC Analysis of Fluorescently Labeled Glycans

Tony Merry and Sviatlana Astrautsova

1. Introduction

The study of the glycan chains (oligosaccharides) of glycoproteins presents a number of analytical problems that generally make their analysis more difficult than that of the peptides to which they are attached. A number of different analytical techniques have been applied to the study of glycans. Definitive characterization has traditionally been performed using nuclear magnetic resonance spectroscopy (NMR) and this still remains the only way to unequivocally assign the structure to novel glycans, but the techniques do require relatively large amounts of material (generally in the milligram range). In addition, access to the sophisticated equipment and the expertise required for the interpretation of data mean that the technique is available only to specialized dedicated laboratories. Another technique, which has also been applied extensively to studies of glycan structure, is that of mass spectrometry in various forms. A number of structures have been solved by means of fast atom bombardment (FAB-MS) ***(1,2)*** although generally further information from linkage analysis by gas chromatography-MS of permethylated alditol acetates (PMAA ***(3–5)***) or by use of specific exoglycosidase enzymes ***(6)*** is required. Other techniques for mass spectrometric analysis are becoming more widely used as a result of recent developments in instrumentation ***(7–9)***. Both matrix-assisted laser desorption mass spectrometry with time-of-flight detection ***(8)*** (MALDI-TOF) and electrospray coupled to post source decay ***(9)*** to produce fragmentation ions are now becoming routinely used. The instrumentation is still expensive, however, and requires skilled operation and interpretation.

Another technique that is also used is capillary electrophoresis, which can offer rapid characterization but that can also be difficult to optimize ***(10)***. Polyacrylamide gel electrophoresis of fluorescently labeled glycans, a technique

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that requires no expensive instrumentation or specialized expertise, may also be used for screening or profiling. Although this is very useful for routine screening of a large number of samples, detailed characterization or sequencing of complex mixtures is problematic.

High-performance liquid chromatography (HPLC) techniques with fluorescent detection offer a compromise in that they give the capability of full characterization of complex glycan mixtures relatively quickly, and although they do require good instrumentation this is considerably less expensive than mass spectrometry or NMR. HPLC is therefore the method of choice for routine analysis of glycosylation of protein, which have glycans of a type that have been previously characterized. Even when the glycan type is novel it is a technique that is compatible with others that can give further structural information, in particular mass spectrometry with LC-MS techniques now becoming fully integrated. The development of HPLC has presented several problems, which are unique to glycan analysis. In particular they generally do not possess a strong chromophore or fluorophore for detection. In addition they may not be charged and different glycans may have very similar composition and physicochemical properties. Therefore, their study has required the development of techniques for their derivatization and also for their separation, which differ from those used for peptides.

When the glycans are released with a free reducing terminus, by either hydrazinolysis or endo-glycosidase, or endo-glycopeptidase enzymes, they may be derivatized by the relatively simple reaction of reductive animation ***(11)***. It is desirable that the derivatization is nonselective to achieve quantitative analysis and it should not cause structural changes such as desialylation. The incorporation of tritium into the C1 position of the reducing terminal monosaccharide fulfils these requirements most effectively, and many studies have been performed by this technique ***(12–14)***. This technique does, however, require the use of relatively large amounts or radioactivity and the use of scintillation counting for high sensitivity work and is not widely used now. Fluorescent labels may also be introduced into the C1 position by similar reductive amidation reactions and a number of these have now been described including 2-amino pyridine ***(15–17)***, 2-aminoacridone ***(18)***, 3-(acetylamino)-6-aminoacridine (AA-Ac) ***(19)***, 2-aminoanthanilic acid ***(20,21)***, and 2-aminobenzoic acid (2-AB) ***(20)***.

A number of chromatographic systems have been developed for separation including gas–liquid chromatography ***(22)*** size-exclusion chromatography on polyacrylamide based beads, notably the BioGel P4 series ***(24,24)***, ion-exchange chromatography on standard matrices ***(25)***, and the development of specialized matrices for anion-exchange (high-performance anion-exchange chromatography [(HPAEC]) ***(26–29)***. They have been used in a large number of studies on protein glycosylation but these techniques have certain drawbacks. Low-pressure size-exclusion chromatography requires great care in column packing

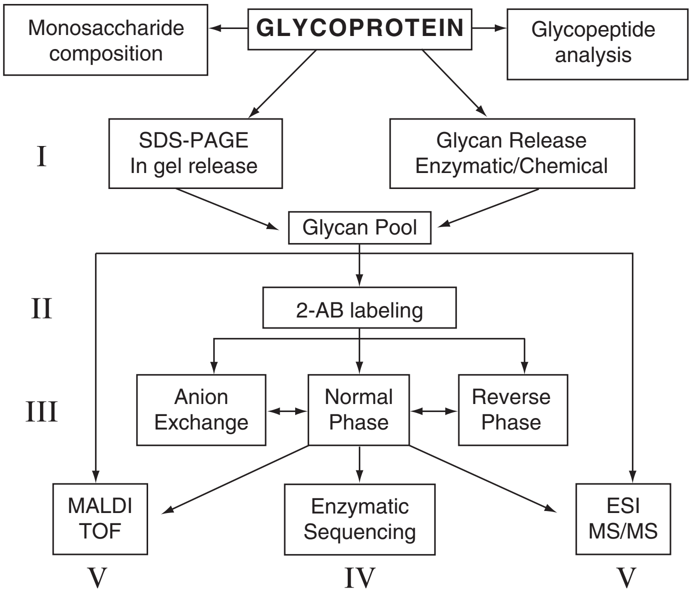
Monosaccharide composition
GLYCOPROTEIN
Glycopeptide analysis
I
SDS-PAGE In gel release
Glycan Release Enzymatic/Chemical
Glycan Pool
II
2-AB labeling
III
Anion Exchange
Normal Phase
Reverse Phase
MALDI TOF
Enzymatic Sequencing
ESI MS/MS
V
IV
V

Fig. 1. The steps involved in complete analysis are shown diagrammatically. The Roman numerals relate to the stages given in the Introduction.

and the run times are long, HPAEC requires the use of high-pH and high-salt buffers that have to be removed before further analysis of separated glycans, such as exoglycosidase sequencing. The use of glycans labeled with a fluorescent tag such as 2-AB ***(20)*** and separated on suitable HPLC matrices ***(30,31)*** provides a convenient and sensitive means of profiling and sequencing of glycoprotein glycans ***(30,32,33)*** that can now be performed in many laboratories.

The analysis of glycans by this technique proceeds in a number of stages:

1. Release of glycans from the glycoprotein.
2. 2-AB labelling of the glycans.
3. Initial HPLC profiling.
4. Enzymatic sequencing of glycans.
5. Conformation of structures by mass spectrometry.

This approach is summarized in **Fig. 1**. The glycan release techniques may be adapted according to the source and purity of the proteins under study.

2. Materials

2.1. Equipment

1. HPLC system: High-pressure mixing two-solvent system capable of delivering 0.1–1.0 mL/min with shallow gradient (*see* **Note 1**).
2. Solvent degasser: Additional solvent degasser aids reproducibility.

3. Detector fluorescent detector capable of excitation at λ_{330nm} and emission at λ_{420nm} (*see* **Note 2**).
4. Computer system for data collection and analysis.
5. Data acquisition software, for example, Waters Millenium™.
6. Curve-fitting software, Microsoft Excel™ or Proprietary software from Instrument Vendor (*see* **Note 3**).

2.2. Reagents

1. Anhydrous hydrazine was prepared by distillation from reagent grade hydrazine (Pierce Aldrich 21515-1) by mixture with calcium oxide and toluene as previously described ***(34)*** (*see* **Note 4**). This is available commercially from Ludger Ltd, Abingdon, UK
2. Acetic anhydride (ACS reagent, Sigma).
3. Anion-exchange resin: Bio-Rad AG50 X12.
4. Acetonitrile: HPLC grade with low background fluorescence such as E Chromosolv® (Reiedel-de-Haën, from Sigma).
5. Formic acid Aristar grade (BDH).
6. 26% Extra pure ammonia solution (Reiedel-de-Haëen, from Sigma).
7. Water MilliQ or equivalent, sub boiling point double distilled water (for mass spectrometric analysis).
8. Whatman no. 3 chromatography paper.
9. 2-Aminobenzamide labelling kit (*see* **Note 5**).
10. Pro-Mem Filter, 0.45 µm cellulose nitrate (R. B. Radley and Co. Ltd., Shire Hill, Saffron Walden, Essex, UK).
11. 3-([3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) detergent buffer: 50 m*M* ammonium formate, pH 8.6, 1.29. w/v CHAPS, 0.1 *M* EDTA.

2.3. Enzymes

1. "PNGase F" is available from Ludger Ltd, Abingdon, UK) PNGQA-BioTM (PNGase F Peptide-N4-(acetyl-ß-glucosaminyl)-asparagine amidaseN-Glycosidase F.)
2. Exoglycosidase enzymes

Sequencing grade enzymes were obtained from Prozyme (Palm Desert, California – distributed in Europe by Ludger Ltd (Abingdon) unless specified and were used with the buffers supplied as shown in **Table 1**. The following enzymes were used for exoglycosidase sequencing and given the abbreviations shown below along with their specificities.

a)	ABS	α2–6 + 2,3 Specific sialidase (Arthrobacter ureafaciens).
b)	NDVS	α2–3 Specific sialidase (Newcastle disease virus).
c)	BTG	β1–3 (>β1,4 or 6) Specific galactosidase (bovine testes).
d)	SPG	β1–4 Specific galactosidase (*Streptococcus pneumoniae*).
e)	AMF	α1–3 Specific fucosidase (almond meal).
f)	BKF	α1–6 (>α1,2 or 3 or 4) Specific fucosidase (bovine kidney).

g)	JBH	β1–2,3, 4 or 6 Specific hexosaminidase (GlcNAc or GalNAc, Jack Bean).
h)	SPH	β1,3 or 6 to Gal β1,2,3 or 6 to Man *N*-acetylglucosaminidase (*Streptococcus pneumoniae*).
i)	JBM	α1–2, 1–3, 1–6 Specific mannosidase (Jack Bean).
j)	HPM	β1–4 Specific mannosidase (*Helix pomatia*).

2.4. Standards

1. Partially hydrolyzed dextran (glucose oligomer standard) (Ludger Ltd, Abingdon UK).
2. Arabinose oligomers (monomer to octamer are available from Dextra Labs, Reading, UK).
3. 2-AB labeled glycan standards, all available from Ludger Ltd (Abingdon UK):
   a. Biantennary galactosylated.
   b. Biantennary galactosylated (with core 1,6-linked fucose).
   c. Biantennary galactosylated (with bisecting GlcNAc).
   d. Biantennary sialylated.
   e. Triantennary.
   f. Tetraantennary.
   g. Oligomannose (Man 5,6,7,8,9).
   h. Type 2 core *O*-glycan.

3. Methods

3.1. Glycan Release

3.1.1. Enzymatic Release with PNGaseF from Glycoproteins in Solution (Method from ***ref. 35*** *)*

1. PNGaseF solution at 1000 U/mL (Boehringer Mannheim).
2. Isolate the glycoprotein according to your usual procedures.
3. The sample should be relatively salt free and contain no extraneous carbohydrates (e.g., Sepharose-purified material contains large amounts of free carbohydrate that should be removed by dialysis using a 15–18,000 mol wt cut-off membrane)
4. If the volume of the glycoprotein solution required is >100 μL, dry the glycoprotein in a 1.5-mL microcentrifuge tube. Generally 50–200 μg of glycoprotein is required.
5. The incubation of a control glycoprotein with known glycosylation alongside experimental samples is recommended (*see* **Note 6**).
6. Proceed with the enzymatic digestion as described in **Subheading 3.1.2**.
7. Store remaining glycoprotein at 4°C for future use.
8. Dissolve sample in 50 μL of 50 m*M* ammonium formate, pH 8.6, 0.4% sodium dodecyl sulfate (SDS).
9. Incubate for 3 min at 100°C.
10. Cool and add 50 μL of CHAPS detergent buffer.
11. Add 1 U of PNGaseF (1 μL).
12. Incubate for 24 h at 37°C (add 5 μL of toluene to prevent bacterial growth).

Table 1
Incubation Conditions for Exoglycosidase Enzymes

Enzyme	Specificity	Buffer	Concentration & Incubation Time*
Sialidase Arthrobacter Ureafaciens EC	α2-3, 6, 8 sialic acid	50mM sodium acetate pH 5.5	1μU/μl 3-16hrs
Sialidase From Newcastle Disease virus Recombinant EC 3.2.1.26	α 2,3 sialic acid only	50mM sodium phosphate pH 6.0	1μU/μl 16hrs
β-galactosidase Bovine Testes EC	β1,3 or 4 Gal	100 mM citrate-phosphate pH 5.0	1mU/μl 3-16hrs
β-galactosidase Streptomyces Pneumoniae EC	β1,3 Gal	100mM Sodium Acetate pH 6.0	80μU/μl 16hrs
β-N acetylhexoseaminidase Recombinant	β1,3 or 4 GlcNAc or GalNAc	100mM Na citrate-phosphate pH 6.0	10μU/μl 16hrs
β-N-acetylglucosaminidase	β1,3 4 GlcNAc	100mM Na citrate/ phosphate	120μU/μl 16hrs pH 6.0

α-fucosidase	α1-4, 6	50 mM NaAc pH 5.0	1mU/μl
Almond Meal	Fuc		16hrs
α-fucosidase	α1–3,	100 mM Na citrate pH 6.0	1mU/μl
Bovine Kidney	Fuc	16hrs	
α-mannosidase	α1-2, 4	100 mM sodium acetate, 2 mM Zn, pH 5.0	67 mU/μ l
Jack Bean	Man		Requires 2× 16hrs incubations
Endo β-galactosidase	β1-3,4 Gal in poly N-acetyl lactosamine	50mM	100μU/μl
B. fragilis		Sodium Acetate pH 5.8	3 hrs

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[*] It should be noted that for most of these exoglycosidases the enzyme concentration and the incubation times have not been optimised for the type of glycan or the amount used in studies with fluorescent glycans. The conditions are generally those which can be expected to give complete digestion of the particular linkages. In practice lower enzyme concentrations and shorter incubation times could often be used. It is advisable to check any enzymes against known standard glycans of different types before use in sequencing.

13. Remove 5 µL and analyze the reaction mixture by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
14. If sample is completely deglycosylated proceed with step otherwise continue with incubation (*see* **Note 8**).
15. Filter samples through protein binding membrane or perform gel filtration.
16. Dry sample in vacuum centrifuge.

3.1.2. Enzymatic Release with PNGaseF from Glycoprotein Bands in SDS-PAGE Gels

Suitable for analysis of low microgram amounts of protein or for unpurified proteins separated by SDS-PAGE or 2-dimensional electrophoresis. Method of Kuster et al. ***(36)***. See Royle et al. ***(37)*** for later modifications

1. Destain Coomassie Blue stained gels in 30% methanol–7.5% acetic acid.
2. Cut out gel pieces with band of interest using a washed scalpel blade, keeping pieces as small as possible.
3. Put into 1.5-mL tubes and wash with 1 mL of 20 m*M* $NaHCO_3$, pH 7.0, twice using a rotating mixer.
4. Add 300 µL of $NaHCO_3$, pH 7.0.
5. Add 20 µL 45 m*M* dithiothreitol (DTT).
6. Incubate at 60°C for 30 min.
7. Cool to room temperature and add 20 µL of 100 m*M* iodoacetamide.
8. Incubate for 30 min at room temperature in the dark.
9. Add 5 mL of acetonitrile–20 m*M* $NaHCO_3$, pH 7.0.
10. Incubate for 60 min to wash out reducing agents and SDS.
11. Cut gel into pieces of 1 mm^2.
12. Place in a vacuum centrifuge to dry.
13. Prepare PNGaseF solution at 1000 U/mL (Boehringer Mannheim): Add 30 U 30 µL of PNGaseF in 20 m*M* $NaHCO_3$, pH 7.0.
14. Allow gel to swell and then add a further 100-µL aliquot of buffer.
15. Incubate at 37°C for 12–16 h.

3.1.3. Release by Hydrazinolysis

Suitable for analysis of *N*- or *O*-linked glycans where the amount of protein is limited, where steric hindrance to enzymatic release is known, or where selective release of glycans by enzymatic means is suspected.

3.1.3.1. PREPARATION OF SAMPLES FOR HYDRAZINOLYSIS

Desalt and dry the samples completely as follows (*see* **Note 8**).

1. Dissolve the sample in 0.1% trifluorocetic acid (TFA) in as small a volume as possible.
2. Set up dialysis at 4°C. A microdialysis system is recommended.
3. Dialyze for a minimum of 48 h in a microdialysis apparatus fitted with a 6000–8000-Dalton cut-off membrane.

4. Recover sample from dialysis membrane. Wash membrane with 0.1% TFA to ensure recovery.
5. Transfer to a suitable tube for hydrazinolysis (*see* **Note 9**).
6. Lyophilize sample for 48 h.
7. For *O*-glycan analysis further drying is recommended (*see* **Note 10**).
8. Remove sample from lyophilizer immediately prior to addition of hydrazine.

3.1.3.2. Hydrazinolysis Procedure

Suitable for analysis of *N*- and *O*-linked glycans when expertise and equipment for the procedure are available ***(34)***.

1. Remove tubes from drying immediately prior to hydrazine addition.
2. Flush tube with argon, taking care not to dislodge lyophilized protein.
3. Rinse dried syringe fitted with stainless steel needle with anhydrous hydrazine.
4. Take up fresh hydrazine and dispense onto sample. A 0.1-mL volume of hydrazine is sufficient to dissolve up to 2 mg of glycoprotein. For larger amounts add more hydrazine.
5. Flame seal the tube. (note reaction may be performed in suitable air tight vials – e.g.
6. Gently shake tube—the protein should dissolve.
7. Place in an incubator (do not use water bath).
8. For release of *N*-linked glycans incubate at 95°C for 5 h; for *O*-glycan release incubate at 60°C for 6 h.
9. Allow to cool and remove hydrazine by evaporation.
10. Add 250 µL of toluene and evaporate. Repeat 5×.
11. Place tube on ice and add 100 µL of saturated sodium bicarbonate solution.
12. Add 20 µL of acetic anhydride.
13. Mix gently and leave at 4°C for 10 min.
14. Add a further 20 µL acetic anhydride.
15. Incubate at room temperature for 50 min.
16. Pass solution through a column of Dowex AG50 X12 (H^+ form)—0.5 mL bed volume.
17. Wash tube with 4 × 0.5 mL of water and pass through a Dowex column.
18. Evaporate solution to dryness. This should be done in stages by redissolving in decreasing volumes of water.
19. Prepare 50 × 2.5 cm strips of Whatman no. 1 chromatography paper (prewashed in water by descending chromatography for 2 d).
20. Spot sample on strip.
21. Perform descending paper chromatography for 2 d in 4:4:1 by vol butanol–ethanol–water for *N*-glycans and 8:2:1 1 by vol butanol–ethanol–water for *O*-glycans.
22. Remove strip from tank and allow all traces of solvent to evaporate.
23. Cut out region of strip from −2 cm to +5 cm from application.
24. Roll up cut chromatography paper and place in a 2.5-mL nonlubricated syringe.
25. Fit a 0.45 µm PTFE filter to syringe.
26. Add 0.5 mL of water and allow to soak into paper for 15 min.
27. Fit syringe plunger and force solution through filter.
28. Wash filter with water 4×, and pass through filter.
29. Evaporate sample to dryness, dissolve in 50 µL of water, transfer to microcentrifuge tubes, and store at −20°C until required.

3.2. Glycan Labeling

Fluorescent labelling with 2-aminobenzamide was performed as described by Bigge et al. ***(20)*** using the kit provided by Ludger Ltd.(Abingdon, UK). The procedure is as follows:

1. Take an aqueous solution of glycan that should contain a minimum of 0.1 pmol and a maximum of 50 pmol of glycans. The volume should be no more than 50 µL.
2. Place in a 0.5-mL microcentrifuge tube and dry down in centrifugal evaporator.
3. Prepare labelling solvent by addition of 150 µL of glacial acetic acid to 100 µL of dimethyl sulfoxide (DMSO). Add 100 µL of the acidified dimethyl sulfoxide solvent to 2-aminobenzamide to make a 0.25 *M* solution. Mix well to dissolve the 2-AB (may require gentle warming).
4. Add all this solution to sodium cyanoborohydride to make a 1.0 *M* solution.
5. Mix well for 5 min to dissolve the reductant.
6. Add 5 µL of the labelling reagent to the dried sample.
7. Mix well and centrifuge briefly.
8. Incubate at 65°C for 1 h and mix well. Incubate for a further 2 h.
9. Cool the labelling mixture on ice.
10. Remove free label by either technique given in **Subheadings 3.2.1** and **3.2.2**.

3.2.1. Removal of Free 2-AB Label by Ascending Paper Chromatography

1. Cool the 2-AB reaction mixture in freezer.
2. Apply all 5 µL of sample to a point in centre of strip 1 cm from the bottom.
3. Allow to dry for 2 h.
4. Perform ascending chromatography for 1 h in acetonitrile.
5. Examine strip under UV to see if the spot of free dye has migrated to the top of the strip.
6. If it is not at the top continue chromatography until it is.
7. Dry the paper completely.
8. Cut out the origin on the strip.
9. Place in a 2.5-mL syringe fitted with a 0.45-µm PTFE filter.
10. Apply 0.5 mL of water and leave for 15 min.
11. Push water through the filter.
12. Wash twice with a further 0.5 mL of water.
13. Dry down labeled sample in a vacuum centrifuge.

3.2.2. Removal of 2-AB Label on Filter Disks

1. Before use wash Whatman no. 1 filter paper in 500 mL of MilliQ water. Place filter paper in a beaker and add water. Leave for 15 min at room temperature. Decant water. Repeat this 4 times.

2. Dry paper in a 65°C over for 2 h. The paper may be stored at room temperature after this step.
3. Cut 6-mm discs from washed paper.
4. Place discs in a Bio-Rad disposable column. For a sample of < 10 µg of glycoprotein use two discs; for more use five discs).
5. Add 2 mL of water and leave for 5 min with columns capped.
6. Uncap columns and wash with another 4 × 2 mL of water (*see* **Note 11**).
7. Wash with a further 5 × 2 mL of 30% acetic in water (*see* **Note 11**).
8. Cap column and add 2 mL of acetonitrile.
9. Leave for 5 min and uncap the column.
10. Wash with a further 2 mL of acetonitrile just before applying sample; cap column.
11. Remove incubation vial and put in freezer for 5 min to cool down.
12. Centrifuge the tube and spot all the sample onto the disc.
13. Leave for 15 min and rinse tube with 100 µL of acetonitrile and add to disc. Leave for 5 min.
14. Add 2 mL of acetonitrile—uncap column and discard wash.
15. Wash with a further 4 × 2 mL of acetonitrile.
16. Place syringes (2.5-mL Fortuna) fitted with a 0.45-µm filter and stoppers, under the filter.
17. Elute glycans with 4 × 0.25 mL of water (*see* **Note 11**).
18. Dry sample down for analysis.

Note: *A kit employing a proprietary separation membrane for separation of free 2-AB from glycans (LudgerCleanTM EB Cartridges) is now available from Ludger Ltd (Abingdon, UK)*

3.3. Normal Phase Chromatography

The following conditions are recommended:

1. Buffers:

Ammonium formate: 50 m*M* formic acid adjusted to pH 4.4 with ammonia solution.

Acetonitrile: HPLC grade;

2. Column: GlycoSep N™ column (Ludger Ltd) or TokoHas TSK-amide 80.
3. Gradient:
4. Sample loading:
   a. The sample should be loaded in 80% acetonitrile–20% water (v/v). In practice take 20 µL of the sample in water and add 80 µL of acetonitrile.
   b. Inject 95 µL of the sample.
   c. A standard dextran should be included with all sample runs.
   d. **Fig. 2** shows a typical separation of human serum IgG with dextran ladder calibration.

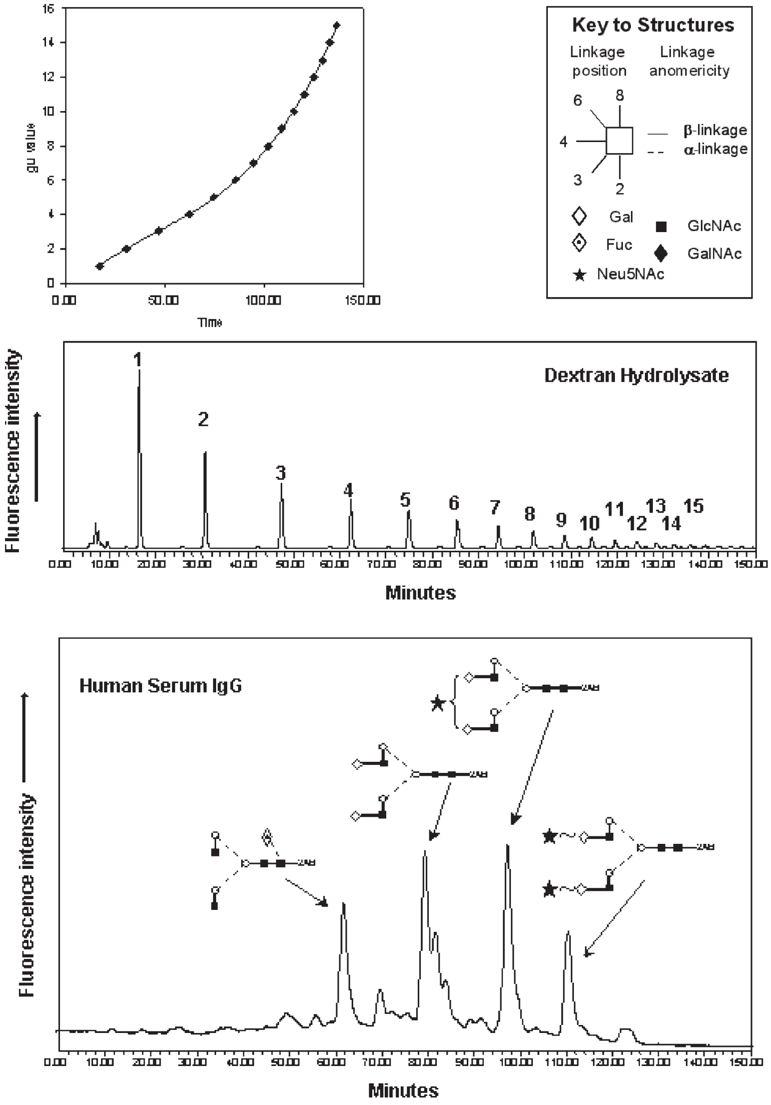

Fig. 2. Analysis of IgG N-glycans released by hydrazinolysis on normal phase HPLC. The top panel shows the calibration curve for conversion of retention time into glucose units based on a third order polynomial. The *middle panel* shows the separations of glucose oligomers from the dextran ladder. The *lower panel* shows the separation of the fluorescently labeled glycans from IgG with structures corresponding to the major peaks indicated. Software (GlycoBase) for this purpose is now available on the Internet as part of the EuroCarbDB project (http://www.eurocarbdb.org/) (*see* **Note 3**).

a. Startup method run time, 30 min.				b. Separating method run time, 180 min			
Time (min)	Flow (ml/min)	A (%)	B (%)	Time (min)	Flow (ml/min)	A (%)	B (%)
0	0	20	80	0	0.4	20	80
04	1.4	20	80	152	0.4	058	42
08	1.4	95	05	155	0.4	100	00
13	1.4	95	05	157	10.	100	00
16	1.4	20	80	162	10.	100	00
25	1.4	20	80	163	10.	020	80
26	0.4	20	80	177	10.	020	80
60	0.4	20	80	178	0.4	020	80
61	0	20	80	260	0	2200	80

5. Data analysis:
   a. The elution times of all peaks in the dextran ladder should be recorded.
   b. To assign glucose unit values a polynomial fit should be applied to the data to generate a standard curve. A third-order polynomial will generally give a good fit (*see* **Fig. 2**).
   c. The glucose unit values of sample peaks may then be calculated (*see also* **Note 3**).
   d. Published values of glucose unit values for a wide series of glycans may be used to give an indication of possible structures present. These are now available on the Internet as part of the GlycoBase software developed by NIBRT, Dublin (http://glycobase.ucd.ie/cgi-bin/public/glycobase.cgi) for the EUROCarbDB project (http://www.eurocarbdb.org/)

3.4. Reverse-Phase Chromatography

1. Buffers:
   a. ammonium formate–triethylamine 50 m*M* formic acid adjusted to pH 5.0 with triethylamine.
   b. Acetonitrile: HPLC grade; *see* **Subheading 2.2.**
2. Column: Reverse-phase column GlycoSep™ R—Ludger Ltd (Abingdon, UK) or equivalent.
3. Sample loading:

a. Startup method run time, 30 min

Time	Flow	A	B
00	0.05	05	95
05	100.	05	95
10	100.	05	95
20	100.	05	95
21	100.	95	05
28	100.	95	05
29	0.50	95	05
90	0.50	95	05
91	00.0	95	05

b. Separating method run time, 180 min

Time	Flow	A	B	
000	0.5	95	05	
030	0.5	95	05	
160	0.5	85	15	
165	0.5	76	24	
166	1.5	05	95	
172	1.5	05	95	
173	1.5	95	05	
178	1.5	95	05	
179	0.5	95	05	
220	0.5	95	05	
221		0	95	5

a. The sample should be loaded as a aqueous solution. A volume of 95 µL of sample should be loaded.
b. A standard of an arabinose oligomers (available from Dextra Labs, Reading) should be included with all sample runs.
4. Data analysis:
a. The data may be analyzed in a similar way to that of normal phase but using the values for the arabinose ladder.
b. Typical values for the AU units for a number of structures are available *(37)*.
c. It can often be helpful to compare normal and reverse-phase profiles for the same glycans as shown in **Fig. 3**. The different principles for separation are illustrated by the differences in separation on the two systems. By combining both types of analysis all four structures can readily be identified, for example, the separation of A2G2F and A2G2FB.

3.5. Weak Anion-Exchange Chromatography (WAX)

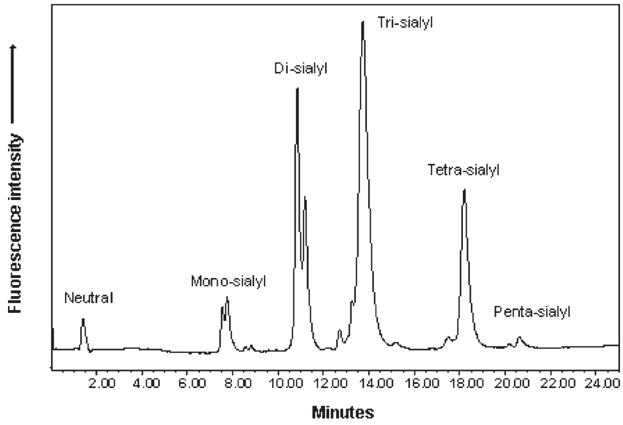

Fig. 3. Analysis of fetuin on Weak Anion Exchange Chromatography. The separation of glycans possessing from 1 to 5 sialic acids is shown.

1. Buffers:
a. 500 m*M* Formic acid adjusted to pH 9.0 with ammonia solution.
b. Methanol water: 10:90 (v/v).
2. Gradient
3. Sample loading
a. The sample should be loaded as an aqueous solution. A volume of 95 µL of sample should be loaded.

a.Startup method run time, 30 min. Separating method run time, 180 min

Time	Flow	A	B	Time	Flow	A	B
000	0	0	0	0	1	00	100
05	10.	05	95	12	1	005	095
20	10.	05	95	50	1	080	020
21	10.	95	05	55	1	100	000
28	10.	95	05	65	1	100	000
29	0.5	95	05	66	2	000	100
90	0.5	95	05	77	2	000	100
91	00.	95	05	78	1	000	100

b. No suitable standards are currently available although it is useful to run a well characterized glycoprotein such as bovine serum fetuin (Sigma).
c. The glycans generally elute on the basis of charge although the size of the glycan also contributes to the position of elution.
d. A typical separation of charged *N*-glycans from bovine serum fetuin is shown in **Fig. 3**.

3.6. Exoglycosidase Digestion of N-Glycans

Digestions are generally performed on the pool of glycans by the application of exoglycosidases under the incubation conditions shown in Table 1 in a series of enzyme arrays of increasing complexity as shown in Table 2.

1. The amount required for each digestion can be judged from the previous profiling run, but in general 100 fmol of glycan is detectable. In practice more may

Table 2
Typical Arrays for N-Glycan Sequencing

Array	Vol enzyme	Vol buffer	Water
ABS	1	2	7
ABS BTG	1 2	2	5
ABS BTG BKF	1 2 1	2	4
ABS BTG BKF AMF	1 2 1 1	2	3
ABS BTG BKF AMF JBH	1 2 1 1 2	2	1
JBM (high)	10		
JBM (low)	0.8		9.2

be used with the enzyme concentrations given below if sufficient material is available.

2. Pipette no more than 50 μL of solution into a 0.5-mL microcentrifuge tube.
3. Evaporate glycan solutions to dryness in a vacuum centrifuge.
4. Add 2–5 μL of enzyme solution and 2 μL of incubation buffer.
5. Make the total volume up to 10 μL with water.
6. Incubate for 16–24 h at 37°C.
7. Cool and load the digestion mixture onto a protein binding filter (Microspin 45).
8. Leave for 15 min at room temperature.

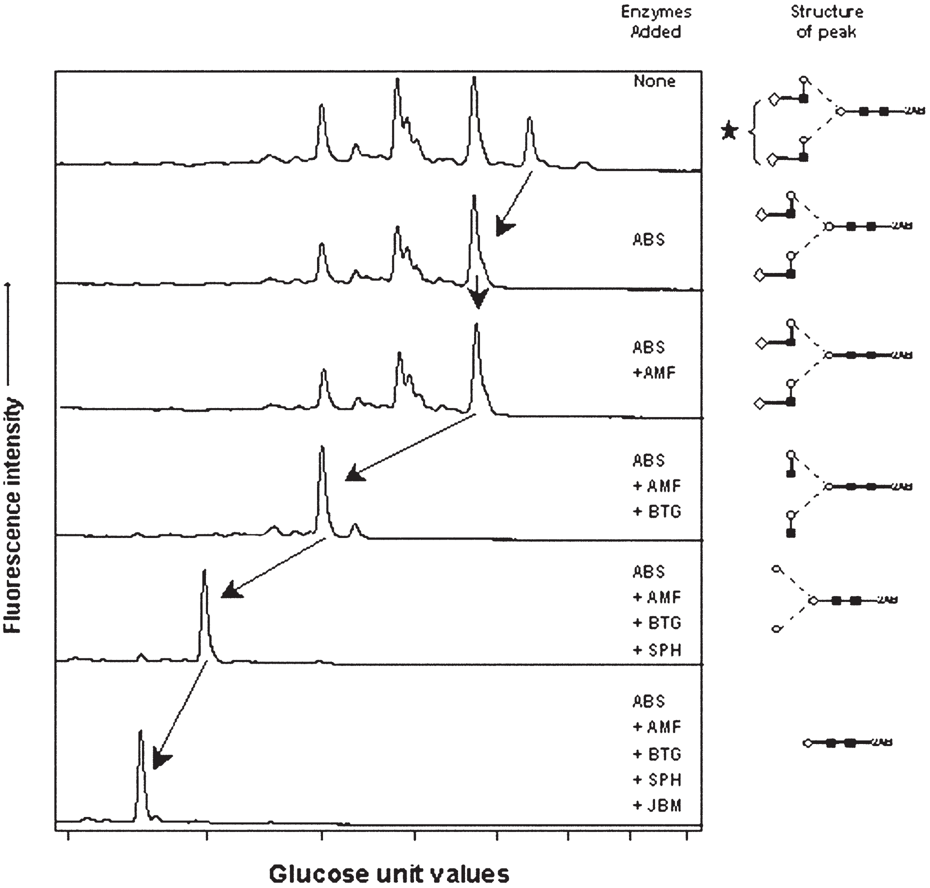

Fig. 4. Exoglycosidase digestion of IgG glycans monitored on normal phase column normal phase HPLC. Exoglycosidase abbreviations ABS – α 2,6 + α 2,3 specific sialidase (Arthrobacter Ureafaciens), BTG 1,2 (+ α 1–4) specific galactosidase (Bovine Testes), AMF- α 2,3 specific fucosidase (Almond Meal) SPH- β1,3 (4,6) specific hexosaminidase (Streptococcus Pneumonae) JBM-α-mannosidase (Jack Bean).

9. Centrifuge for 15 min at 5000*g*.
10. Wash tube with 10 µL of freshly prepared 5% acetonitrile in water and load onto filter.
11. Leave for 15 min at room temperature.
12. Centrifuge for 15 min at 5000*g*.
13. Repeat **steps 10–12**.
14. Twenty microliters of sample may be directly analyzed by HPLC if sufficient material is available; if not, then dry down sample and redissolve in 20 µL of water.
15. Example of digestion of *N*-glycans from IgG is shown in **Fig. 4**.

3.7. Exoglycosidase Digestion of O-Glycans

Digestions are generally performed on the pool of glycans; however, the strategy differs from that for *N*-glycans. Digestions should be performed by the following mixtures of glycans where the choice of glycosidases is dictated by the outcome of previous digestions. Since several *O*-glycans may co elute on any matrix the analysis on both normal and reverse phase is recommended. Incubation conditions recommended for common exoglycosidases are shown in Table 1. A detailed description of the procedure and reference values for digestion products may be found at the following web site; http://glycobase.ucd.ie/

1. The samples are prepared for digestion as described in steps 1–3 (*see* **Subheading 3.6**).
2. First perform ABS digestion to detect presence of sialic acid (in any linkage).
3. Perform ABS + BTG digestion to show the presence of galactose.
4. Perform ABS + BKF digestion to show the presence of fucose.
5. Perform ABS + JBH digestion to show presence of *N*-acetylglucosamine or *N*-acetylgalac-tosamine.
6. In each case digestion will be apparent from a shift in peaks. If the digests are analyzed by normal phase and reverse phase then possible identities of structures may be found from the GU and AU values.
7. If digestion with ABS occurs then also digest pool with NDVS to determine if the sialic acid linkage is 2,3.
8. If digestion with ABS + BTG occurs then digest with ABS + SPG to determine if linkage of galactose is 1,4.
9. If digestion occurs with ABS + BKF then digest with ABS + AMF to determine if fucose linkage is 1,3 or 1,4.
10. If digestion with ABS + JBH occurs then digest with ABS + SPH to determine if *N*-acetylglucosamine or *N*-acetylgalactosamine is present.
11. Note that the complete characterization may require repeated digestions as there is no common core sequence as found for *N*-glycans.
12. Example of *O*-glycan sequencing is shown **Fig. 5**.

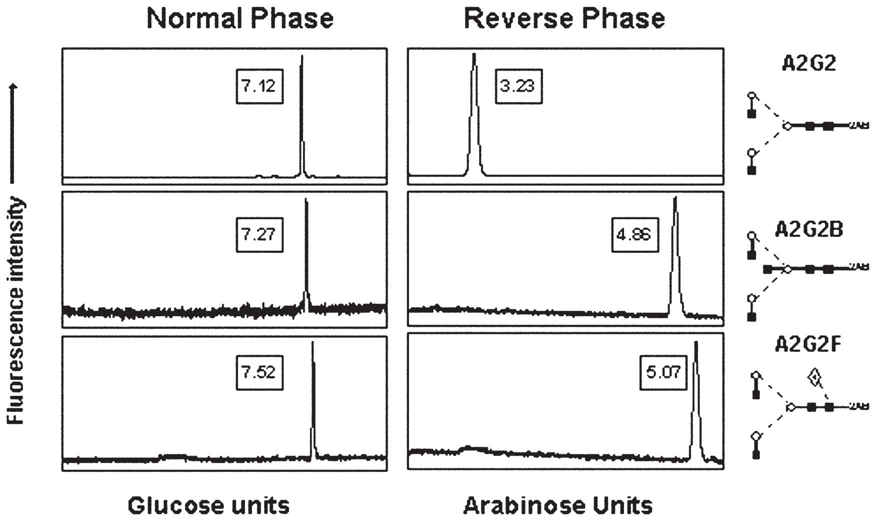

Fig. 5. Analysis of bi-anennary, fucosylated, bisected glycans by reverse phase HPLC – comparison with normal phase HPLC showing complimentarily of separation techniques.

3.7.1. Data Analysis (software for this analysis is now available as pert of the open access EUROCarbDB project (http://www.eurocarbdb.org/).

1. The movement of a peak in an exoglycosidase incubation indicates that it contains the monosaccharides in the linkages removed by that enzyme.
2. A shift in the position of peaks will be seen with decreasing complexity of the chromatogram with increase in the number of enzymes applied.
3. Reference to the last chromatogram in a series will allow identification of the basic core *N*-glycan structure as shown in **Fig. 4**.
4. The last digest in a series will indicate the core glycans present.
5. Working back through the digests, the structures from which a glycan has been removed are identified.

a. In the example the presence of terminal *N*-acetylglucosamine in the structure is shown comparing digest 5 to 4.
b. The presence of terminal galactose is shown by comparing digest 3 to 4.
c. The presence of sialic acid is shown by comparing digest 3 to 1.
d. In this way the structure shown can be assigned to peak A.

6. The presence of other peaks indicates that monosaccharides not covered by the array are present (such as oligomannose) or that residues are substituted by groups such as sulfate or phosphate.
7. An examples of *O*-glycan sequencing is shown in **Fig. 6** with sequential application of glycosidases.

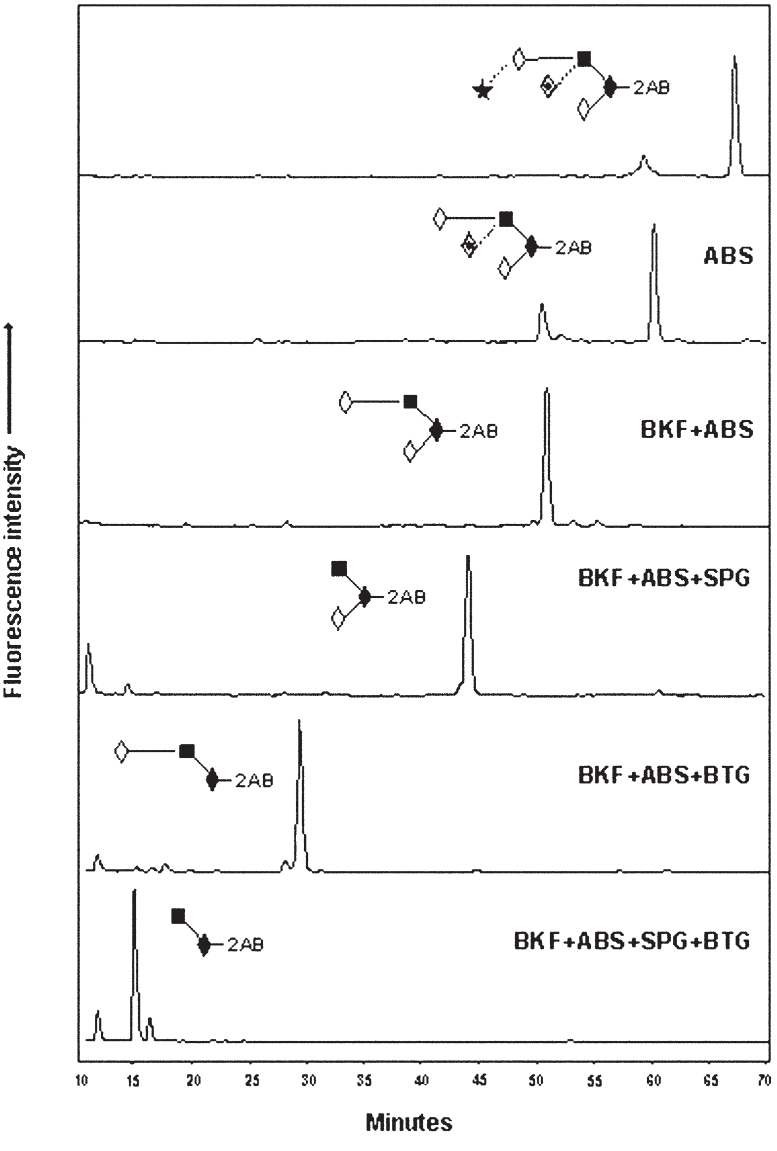

Fig. 6. Example of exoglycosidase sequencing an *O*-glycan on normal phase HPLC. SPG -β1,4 specific galactosidase (*Streptococcus pneumoniae*), other exoglycosidse abbreviations as in **Fig. 4**.

3.8. Mass Spectrometry

Samples may be further analyzed by mass spectrometry (MALDI-TOF or ESI/ MS/MS) for confirmation of structures but generally require further desalting although online systems are now available and are particularly suitable for this kind of analysis. A recent International Study by HUPO (Human Disease Glycolic/Proteome Initiative) compared various techniques for HPLC analysis and showed that the combination of HPLC and Mass Spectrometry allowed sensitive and accurate analysis of N-glycans *(38)*. This study is now being extended to O-glycans. The tools developed by the EuroCarbDB are particularly recommended

1. It is often useful to perform the MS analysis on the glycan pool alongside the HPLC analysis but approx 10× more material is generally required.
2. As all solvents used are volatile, fractions containing peaks from the HPLC run may be collected and dried in a vacuum centrifuge but cleanup may be required (*see* **Note 12**).

4. Notes

1. Waters System is recommended. Hewlett-Packard is also suitable. System with similar specification from other manufacturers may be suitable but must be capable of high-pressure mixing of acetonitrile and aqueous solvents.
2. Jasco or Waters is recommended but other detectors of similar specificity may be suitable.
3. Glycobase is a list of GU values and associated software for analysis of exoglycosidase digestion of glycans that was originally developed at the Oxford Glycobiology Institute and subsequently at NIBRT, Dublin, as part of the EuroCarbDB project. It will form one of the databases available at www.eurocarbdb.org/.
4. Great care should be taking in the handling of hydrazine. Dry hydrazine of suitable quality may be obtained from Ludger Ltd. (Abingdon, UK).
5. Available from Ludger Ltd. (Abingdon, UK).
6. Suitable glycoproteins include ribonuclease B, bovine serum fetuin, and human serum haptoglobulin.
7. The change in position of a band on SDS-PAGE indicates *N*-glycosylation. In some cases incomplete digestion may lead to the production of a series of bands corresponding to partially deglycosylated glycoprotein. Complete release is indicated by conversion to a single band at the lowest molecular weight.
8. The presence of most salts, dyes, or detergents will interfere with hydrazinolysis. The sample must also be completely anhydrous.
9. All glassware used should be soaked in $4\,M$ nitric acid for 4 h and thoroughly washed in water before use.

10. The use of cryogenic drying by placing under high vacuum connected to a trap of charcoal submerged in liquid nitrogen for a period of 72h as described by Ashford et al. ***(34)*** is recommended.
11. It may be necessary to apply slight pressure to the top of the column or to use a suitable vacuum apparatus.
12. Double-distilled (sub boiling point) water should be used for mass spectrometric analysis. Suitable techniques for post-HPLC cleanup and for mass spectrometry by electrospray ***(8)*** and by MALDI ***(9)*** have been described recently.

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The techniques described here are the result of many years of development by previous members of the Oxford Glycobiology Institute, and in particular Dr David Harvey for his mass spectrometry expertise, Dr. Geoffrey Guile for his HPLC expertise and Dr. Taj Mattu who were all involved in the development of the protocols described in this chapter.

Although the group no longer exists at the Glycobiology Institute in Oxford Prof Rudd is continuing development and in particular training all the procedures detailed here at the NIBRT Dublin and the EuroGlycosciences Forum (www.glycosciences.eu) is promoting training in this area.

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Glycoprofiling Purified Glycoproteins Using Surface Plasmon Resonance

Angeliki Fotinopoulou and Graham A. Turner

1. Introduction

1.1. Glycosylation and Its Investigation

The carbohydrate part of glycoproteins can define several of their biological properties, including the clearance rate, immunogenicity, thermal stability, solubility, the specific activity, and conformation ***(1)***. Often, small differences in the composition of the sugar side chains of a glycoprotein can affect the biological properties ***(2)***. Characterization of oligosaccharide structures on glycoproteins is essential for glycoprotein therapeutic products, because inflammatory and systemic responses may arise if glycosylation of the product is different from that of the native substance.

Determination of the precise oligosaccharide profile of a glycoprotein, however, cannot yet be considered as a routine laboratory task. Methods for glycosylation analysis can be generally divided in two major categories, direct methods and methods using lectins. This chapter describes a recently developed method that measures the binding of lectins to glycoproteins using surface plasmon resonance (SPR). Lectins are a class of proteins that bind to carbohydrates in a noncovalent reversible way that does not chemically modify the sugar molecule ***(3)***. The lectin specificity is not absolute, but usually there is one carbohydrate grouping to which a lectin binds with much higher affinity than to other carbohydrate structures. This property makes them ideal for recognizing oligosaccharide structures. Although lectin methods are indirect, they can be used when comparisons are needed. They provide many advantages compared to other methods for investigating carbohydrate structures. There are a large number of lectins available with different and distinct specificities. Lectin methods are also quick and simple ***(4)***.

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1.2. Surface Plasmon Resonance and Lectins

SPR is an optical sensing phenomenon that allows one to monitor biomolecule interactions in real time (**Fig. 1**). The sensor device is composed of a sensor chip consisting of three layers (glass, a thin gold film, and a carboxymethylated dextran matrix); a prism placed on the glass surface of the chip; and a microfluidic cartridge, which controls the delivery of liquid to the sensor chip surface *(5).* When light illuminates the thin gold film, energy is transferred to the electrons in the metal surface causing the reflected light to have reduced intensity at a specific incident angle. This angle of nonreflectance changes as the refractive index in the vicinity of the metal surface changes. The refractive index depends upon the mass on the surface of the gold film. A response of 1000 resonance units (RUs) corresponds to a shift of 0.1° in the resonance angle and represents a change in the surface protein concentration of about 1 ng/mm^2 *(6).*

Because all proteins, independent of sequence, contribute the same refractive index, SPR can be used as a mass detector. A glycoprotein, therefore, can be immobilized onto a sensor chip surface and then be probed by a panel of lectins. The binding or nonbinding of the lectins provides information about the oligosaccharide structures found on the carbohydrate chains. For example, if a glycoprotein has a trimannose core on its *N*-linked chains, it will bind to the lectin concanavalin A (Con A), or if it has terminal α2–3 or α2–6 *N*-acetylneuraminic acid (Neu5NAc) on its oligosaccharide chains it will bind to the Neu5NAc specific lectins *Maackia amurensis* agglutinin (MAA) or *Sambucus nigra* agglutinin (SNA), respectively.

Lectin binding can also be performed after treatment with glycosidases to gain more information about the oligosaccharide structures present. By sequentially treating the immobilized glycoprotein with glycosidases and measuring

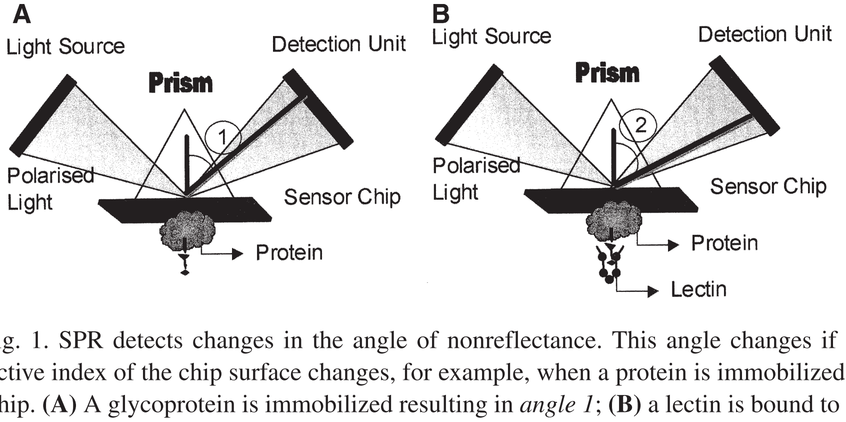

Fig. 1. SPR detects changes in the angle of nonreflectance. This angle changes if the refractive index of the chip surface changes, for example, when a protein is immobilized on the chip. **(A)** A glycoprotein is immobilized resulting in *angle 1*; **(B)** a lectin is bound to the immobilized glycoprotein resulting in *angle 2*.

the binding of a panel of lectins after every treatment it is possible to gain information on the oligosaccharide sequence.

Some lectins will bind to the immobilized glycoprotein only if they are pretreated with sialidase to remove the terminal Neu5NAc. An example of this is peanut agglutinin (PNA), which binds to Galactose β1–3 *N*-acetylgalactosamine groupings on *O*-linked chains after the removal of Neu5NAc.

SPR has been used for investigating the kinetics of the interaction between oligosaccharides and lectins ***(7–9),*** for characterization of fetuin glycosylation ***(10),*** for the determination of agalactoIgG in rheumatoid arthritis patients ***(11),*** and recently, we have developed a method for investigating the glycosylation of recombinant glycoproteins ***(12).***

2. Materials

2.1. Reagents

1. 0.05 *M N*-Hydroxysuccinimide (NHS).
2. 0.2 *M N*-ethyl-*N*' (dimethylaminopropyl)-carbodiimide (EDC).
3. 1 *M* Ethanolamine hydrochloride, pH 8.5.
4. 0.1 *M* HCl.
5. Running buffer (HBS): 10 m*M* 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), pH 7.4, 150 m*M* NaCl, 3.4 m*M* ethylenediaminetetraacetic acid (EDTA), 0.0005% Surfactant P20.
6. 10 m*M* Sodium acetate, pH 4.0.
7. 10 m*M* Sodium acetate, pH 4.5.
8. 10 m*M* Sodium acetate, pH 5.0.
9. 10 m*M* Sodium acetate, pH 5.5.
10. Lectin buffer: 10 m*M* sodium acetate, pH 5.0 with cations 2 m*M* $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CaCl_2$.
11. Lectins used: *MAA, SNA, Datura stramonium Agglutinin (DSA), ConA, PNA, aleuria aurantia Agglutinin (AAA).*

2.2. Equipment

BIAcore 1000 apparatus (Biacore AB, Uppsala) is fully automated and controlled by the manufacturer's software. During the operation of the equipment the output from the chip is displayed on VDU as resonance units (RUs) vs time.

3. Method

The procedure used for lectin/SPR can be divided into four steps. The first three are essential for any SPR experiments and the last one is optional.

1. "Preconcentration" step.
2. Ligand immobilization step.
3. Lectin binding.
4. Enzyme treatment.

3.1. Preconcentration

Before immobilizing a ligand to the chip surface a procedure called "preconcentration" needs to be performed. The latter step is important for efficient chemical immobilization of the ligand. It is accomplished by passing the ligand over the chip surface and utilizing the electrostatic attraction between the negative charges on the surface matrix (carboxymethyl dextran on a CM chip) and positive charges on the ligand at pH values below the ligand pI. Preconcentration is performed at 25°C. The ligand is injected at different pHs and the pH that provides the steepest curve is used (*see* **Note 1**). The detailed sequence of steps is as follows:

1. A continuous flow of HBS buffer is applied at flow rate of 10 mL/min.
2. A 2-min pulse of ligand in 10 m*M* sodium acetate, pH 4.0, is applied.
3. A 2-min pulse of ligand in 10 m*M* sodium acetate, pH 4.5, follows.
4. A 2-min pulse of ligand in 10 m*M* sodium acetate, pH 5.0, follows.
5. A 2-min pulse of ligand in 10 m*M* sodium acetate, pH 5.5, follows.
6. Two 2-min washes with 0.1 *M* HCl follow for regeneration.

3.2. Glycoprotein Immobilization

Choosing the correct matrix for immobilization depends on properties of the glycoprotein to be used and the functional groups on the chip surface. There are various types of chips available, which utilize different immobilization chemistries. Amine coupling is used for neutral and basic proteins ***(13).*** This introduces *N*-hydroxysuccinimide esters into the surface matrix by modifying the carboxymethyl groups of the matrix with a mixture of NHS and EDC. These esters react with the amines and other nucleophilic groups on the ligand to form covalent links.

Optimization of the amount of immobilized ligand is an important factor in SPR measurements and different buffers, ligand concentrations and injection times must be investigated. This procedure is performed at 25°C **(Fig. 2)**.

1. A continuous flow of HBS buffer is applied at flow rate of 5 μL/min.
2. The sensor chip surface is activated with a 7-min pulse of NHS and EDC.
3. Immobilization of ligand is performed by a 7-min pulse of the ligand solution (*see* **Notes 2** and **3**).
4. Deactivation of excess reactive groups on the surface and removal of noncovalently bound material is performed by a 7-min pulse of ethanolamine.
5. Two 4-min pulses with HCl perform regeneration (complete removal of nonbound material).

3.3. Lectin Binding

Lectin binding is performed at 37°C. A continuous liquid flow is applied to the chip and the lectin is injected as a short pulse. If the lectin recognizes a carbohydrate grouping on the glycoprotein then it will bind resulting in an increasing

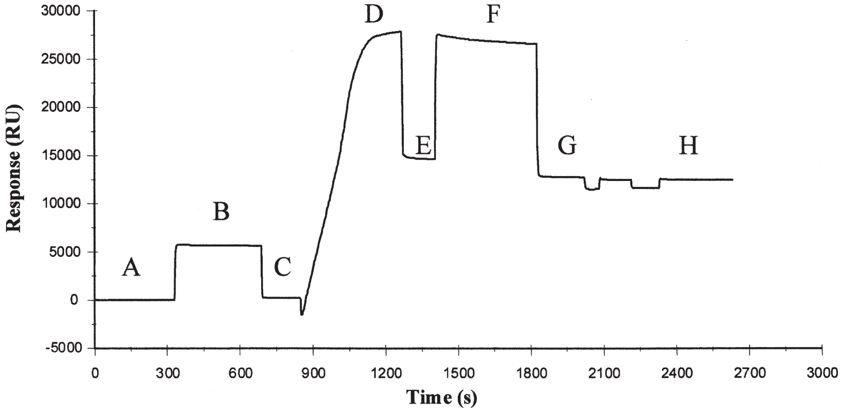

Fig. 2. **(A)** Baseline during continuous buffer flow; **(B)** injection of NHS/EDC to activate the surface; **(C)** baseline after activation; **(D)** injection of ligand; **(E)** immobilized ligand before deactivation; **(F)** deactivation of chip surface using ethanolamine; **(G)** immobilized ligand after deactivation; **(H)** final immobilization level after two HCl washes.

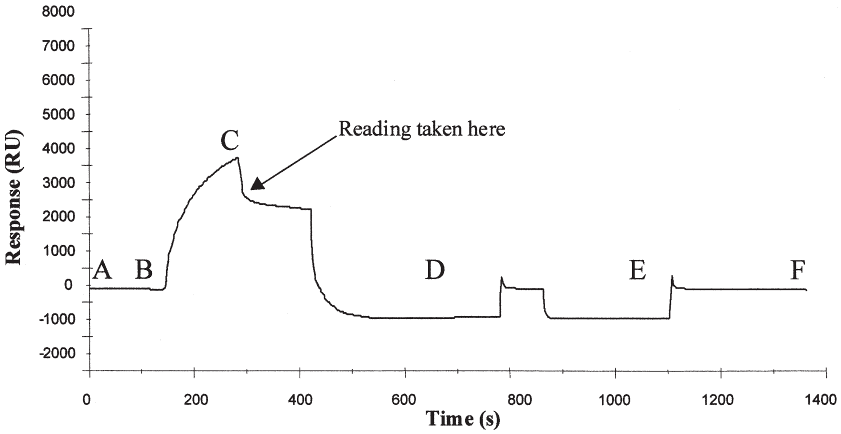

Fig. 3. Typical sensogram of ConA binding. AB, baseline; B, sample injection; BC, association; CD, dissociation; D, E, HCl injections; F, baseline.

RU value. A RU reading is taken approx 15 s after dissociation starts. Bound lectin is removed by passing HCl over the chip and then the binding of another lectin is investigated **(Fig. 3)**.

1. A continuous flow of HBS buffer is applied at flow rate of 5 µL/min.
2. Lectin is injected by a 7-min pulse (*see* **Note 4**).
3. A reading is taken 15 s after dissociation starts (*see* **Note 5**).

4. Lectin is removed by two 4-min pulses with HCl (*see* **Note 6**).
5. **Steps 1–4** are repeated for each lectin and buffer solution.

3.4. Enzyme Treatment

The specificity of lectin binding measured by SPR can be confirmed by enzyme treatment at 37°C with glycosidases. These are usually very specific for one carbohydrate structure. After enzyme treatment lectin binding should not be detected if the interaction was specific. **Figure 4** shows the binding of MAA before and after treatment with neuraminidase. Immobilized glycoproteins can be treated with other enzymes such as galactosidase and PGNase F (*see* **Note 7**).

1. Neuraminidase treatment is performed by a 7-min injection of the enzyme followed by a stop in the flow for 6 h.
2. The flow rate restored back to 5 µL/mL.
3. Regeneration (removal of neuraminidase) is performed by two 4-min pulses with HCl.
4. Lectin injection, reading, and regeneration as in **steps 1–5** of **Subheading 3.3.**
5. The same procedure as described in **steps 1–4** can be repeated for other enzymes until all residues on a sugar chain are chopped off.

4. Notes

1. A preliminary choice of pH for immobilization can be made if the pI of the ligand is known according to the following rule of thumb: for pI > 7, use pH 6, for pI 5.5–7 use 1 unit pH below pI, for pI 3.5–5.5, use 0.5 pH units below pI. For pH range of 4–5.5 10 m*M* acetate buffer is recommended.

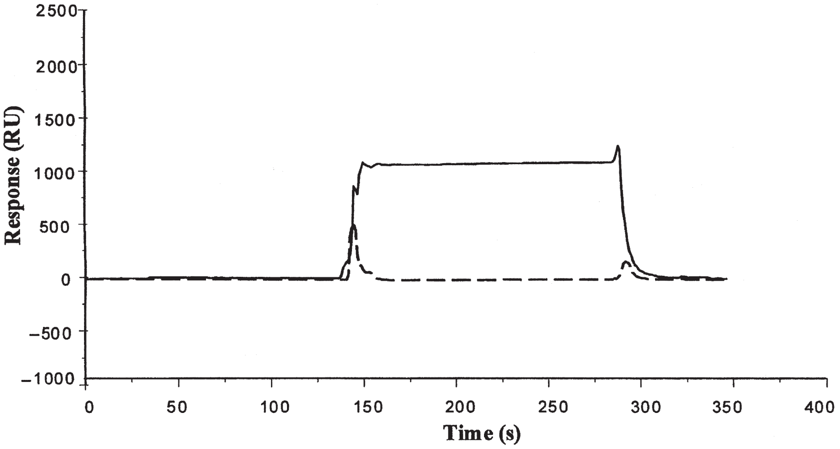

Fig. 4. MAA binding before (*solid line*) and after (*dotted line*) neuraminidase treatment (buffer plot subtracted from both sensograms).

Table 1
Typical Results for Different Glycoproteins with Different Lectins

	ConA	SNA[b]	SNA[a]	MAA[b]	MAA[a]	PNA[b]	PNA[a]	DSA	AAA
IgG	1680	344	50	25	26	24	23	33	71
Fetuin	776	1230	78	624	5	5	80	230	32
Rec1	2768	22	20	10	25	26	30	11	22
Rec2	1203	10	25	325	22	4	297	42	15

[a,b] Before and after neuraminidase treatment. IgG and fetuin were commercial products, while rec1 and rec2 are recombinant glycoproteins. Buffer readings gave values of 20–35 RU. *See* **Note 8.**

2. The time of activation may be modified to regulate the amount of ligand immobilized.
3. For a given pH the ideal ligand concentration is the lowest value that gives maximum preconcentration. Usually, a suitable concentration will be in the range 10–200 µg/mL. A target response is a level of immobilization that gives about 0.07 pmol ligand/mm^2. That corresponds to mol wt/15 RU.
4. Analyte concentration can be chosen between 5 µg/mL and 500 µg/mL.
5. A reading value above 30–40 RU is considered as real binding.
6. After regeneration the baseline should be at the same level as before lectin binding. A drift in the baseline may appear after repeated regeneration, which may be due to loss of Neu5Ac.
7. Different enzyme incubation times may apply depending on the enzyme properties.
8. **Table 1** gives typical results for different glycoproteins with different lectins. The following conclusions can be drawn from these results: IgG has *N*-linked chains (ConA), α2–6 Neu5NAc (SNA) and fucose (AAA) and it does not have any *O*-linked chains (PNA) or any α2–3 Neu5NAc (MAA); Fetuin has *N*- and *O*-linked chains, α2–6 Neu5NAc and α2–3 Neu5NAc, galactose β1–4 *N*-acetylglucosamine (DSA) and no fucose; Rec1 has *N*-linked chains and no fucose, no Neu5NAc, no *O*-linked chains; Rec2 has *N*- and *O*-linked chains, α2–6 Neu5NAc, no α2–3 Neu5NAc and no fucose.

Acknowledgments

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Sequencing Heparan Sulfate Saccharides

Jeremy E. Turnbull

1. Introduction

The functions of the heparan sulfates (HSs) are determined by specific saccharide motifs within HS chains. These sequences confer selective protein binding properties and the ability to modulate protein activities ***(1,2)***. HS chains consist of an alternating disaccharide repeat of glucosamine (GlcN; *N*-acetylated or *N*-sulfated) and uronic acid (glucuronic [GlcA] or iduronic acid [IdoA]). The initial biosynthetic product containing N-acetylglucosamine (GlcNAc) and GlcA is modified by *N*-sulfation of the GlcN, ester (*O*)-sulfation (at positions 3 and 6 on the GlcN and position 2 on the uronic acids) and by epimerization of GlcA to IdoA. The extent of these modifications is incomplete and their degree and distribution varies in HS between different cell types. In HS chains *N*- and *O*-sulfated sugars are predominantly clustered in sequences of up to eight disaccharide units separated by *N*-acetyl-rich regions with relatively low sulfate content ***(3)***.

Sequence analysis of HS saccharides is a difficult analytical problem and until recently sequence information had been obtained for only relatively short saccharides from HS and heparin. Gel chromatography and high-performance liquid chromatography (HPLC) methods have been used to obtain information on disaccharide composition ***(3,4)***. Other methods such as nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy ***(5–9)*** have provided direct sequence information, but are difficult for even moderately sized oligosaccharides and in the case of NMR requires large amounts of material (micromoles). This situation has changed rapidly in the last few years with the availability of recombinant exolytic lysosomal enzymes. These exoglycosidases and exosulfatases remove specific sulfate groups or monosaccharide residues from the nonreducing end (NRE) of saccharides ***(10)***. They can be employed in combination with polyacrylamide gel electrophoresis (PAGE) separations to

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derive direct information (based on band shifts) on the structures present at the nonreducing end of GAG saccharides (***11***; *see* **Fig. 1** for an example).

Integral glycan sequencing (IGS), a PAGE-based method using the exoenzymes, was developed as the first strategy for rapid and direct sequencing of HS and heparin saccharides ***(11)***. Its introduction has been quickly followed by a variety of similar approaches using other separation methods including HPLC and matrix-assisted laser desorption (MALDI) mass spectrometry ***(12–14)***. An outline of the IGS sequencing strategy is given in **Fig. 2**. An HS (or heparin) saccharide (previously obtained from the polysaccharide by partial chemical or enzymatic degradation and purification) is labeled at its reducing terminus with a fluorescent tag. It is then subjected to partial nitrous acid treatment to give a ladder of evenly numbered oligosaccharides (di-, tetra-, hexa-, etc.) each having a fluorescent tag at their reducing end. Portions of this material are then treated

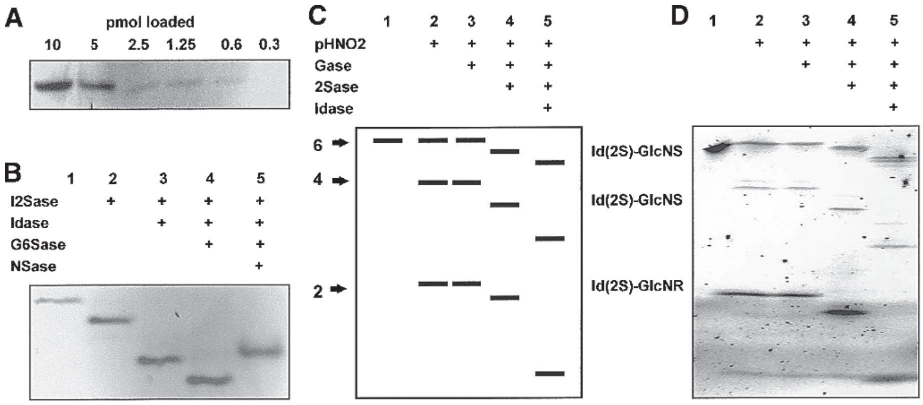

Fig. 1. Basic principles of integral glycan sequencing. (A) Fluorescence detection of different amounts of a 2-AA-tagged heparin tetrasaccharide run on a 33% minigel. (B) Exosequencing of a 2-AA-tagged heparin tetrasaccharide with lysosomal enzymes and separation of the products on a 33% minigel (15 pmol per track). Band shifts following the exoenzyme treatments shown reveal the structure of the nonreducing end disaccharide unit (*track 1*, untreated). I2Sase, iduronate-2-sulfatase; Idase, iduronidase; G6Sase, glucosamine-6-sulfatase; Nsase, sulfamidase. (C) Schematic representation of IGS of a hexasaccharide (pHNO2, partial nitrous acid treatment). (D) Actual example of IGS performed on a purified heparin hexasaccharide, corresponding to the scheme in C, using the combinations of pHNO2 and exoenzyme treatments indicated (*track 1*, untreated, 25 pmol; other tracks correspond to approx 200 pmol per track of starting sample for pHNO2 digest). The hexasaccharide (purified from bovine lung heparin) has the putative structure IdoA(2S)-GlcNSO$_3$(6S)-IdoA(2S)-GlcNSO$_3$(6S)-IdoA(2S)-AMannR(6S). Electrophoresis was performed on a 16-cm 35% gel (from ref. 11, Copyright 1999 National Academy of Sciences, USA).

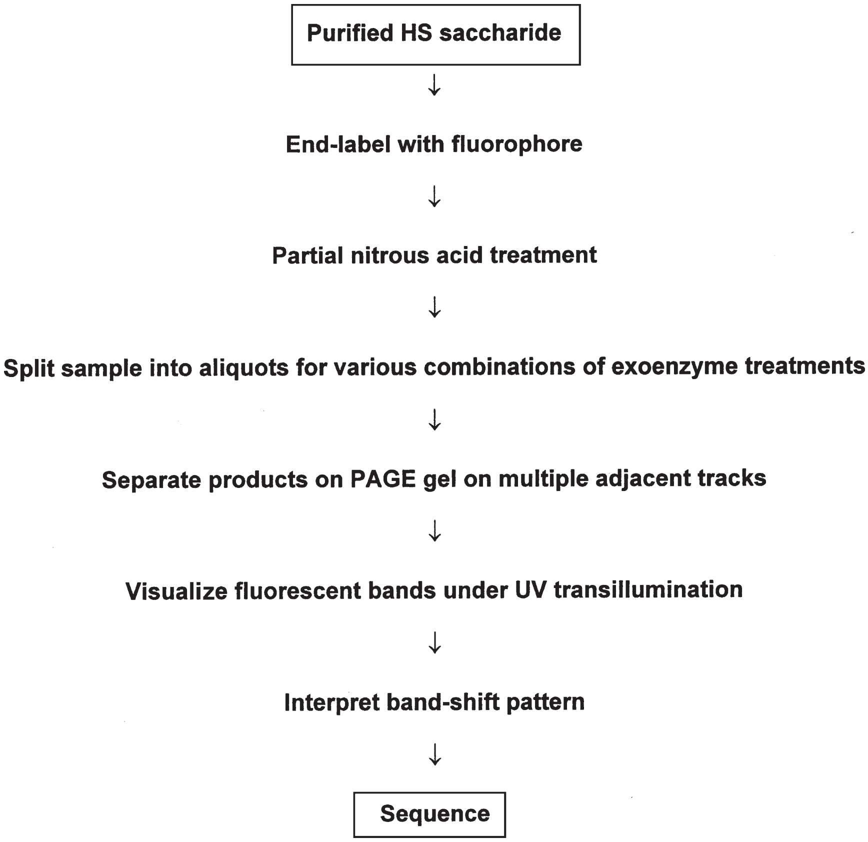
Purified HS saccharide
End-label with fluorophore
Partial nitrous acid treatment
Split sample into aliquots for various combinations of exoenzyme treatments
Separate products on PAGE gel on multiple adjacent tracks
Visualize fluorescent bands under UV transillumination
Interpret band-shift pattern
Sequence

Fig. 2. The IGS sequencing strategy.

with a variety of highly specific exolytic lysosomal enzymes (exosulfatases and exoglycosidases) that act at the nonreducing end of each saccharide (if it is a suitable substrate). The various digests are then separated on a high-density polyacrylamide gel and the positions of the fragments detected by excitation of the fluorescent tag with a UV transilluminator. Band shifts resulting from the different treatments permit the sequence to be read directly from the banding pattern (*see* **Fig. 1** for an example). This novel strategy allows direct read-out sequencing of a saccharide in a single set of adjacent gel tracks in a manner analogous to DNA sequencing. IGS provides a rapid approach for sequencing HS saccharides, and has proved very useful in recent structure–function studies ***(15)***. It should be noted that this methodology is designed for sequencing purified saccharides, not whole HS preparations. An important factor in all sequencing methods is the availability of sufficiently pure saccharide starting material.

HS and heparin saccharides can be prepared following selective scission by enzymic (or chemical) reagents and isolation by methods such as affinity chromatography ***(4)***. Final purification usually requires the use of strong anion-exchange HPLC ***(11,15)***.

2. Materials

1. 2-Aminobenzoic acid (2-AA; Fluka Chemicals).
2. 7-Aminonaphthalene-1,3-disulfonic acid monopotassium salt (ANDSA; Fluka Chemicals).
3. Formamide.
4. Sodium cyanoborohydride (>98% purity).
5. Sodium triacetoxyborohydride (Aldrich).
6. Distilled water.
7. Oven or heating block at 37°C.
8. Desalting column (Sephadex G-25; e.g., HiTrap™ desalting columns, Pharmacia).
9. Centrifugal evaporator.
10. 200 m*M* HCl.
11. 20 m*M* Sodium nitrite (1.38 mg/mL in distilled water; prepare fresh).
12. 200 m*M* Sodium acetate, pH 6.0:27.2 g/L of sodium acetate trihydrate; adjust pH to 6.0 using acetic acid.
13. Enzyme buffer: 0.2 *M* Na acetate, pH 4.5. Make 0.2 *M* sodium acetate (27.2 g/L of sodium acetate trihydrate) and 0.2 *M* acetic acid (11.6 mL/L) and mix in a ratio of 45 mL to 55 mL, respectively.
14. Enzyme stock solutions (typically at concentrations of 500 mU/mL, where 1 U = 1 μmol substrate hydrolyzed per minute). Available from Glyko, Novato, CA.
15. Vortex tube mixer.
16. Microcentrifuge.
17. Acrylamide stock solution (T50%–C5%). **Caution: Acrylamide is neurotoxic.** Wear gloves (and a face mask when handling powdered forms). It is convenient to use premixed *bis*-acrylamide such as Sigma A-2917. Add 43 mL of distilled water to the 100-mL bottle containing the premixed chemicals and dissolve using a small stirrer bar (approx 2 h). The final volume should be approx 80 mL. Store the stock solution at 4°C. Note that it is usually necessary to warm gently to redissolve the acrylamide after storage.
18. Resolving gel buffer stock solutions: 2 *M* Tris-HCl, pH 8.8 (242.2 g/L of Tris base; adjust pH to 8.8 with HCl).
19. Stacking gel buffer stock solution: 1 *M* Tris-HCl, pH 6.8 (121.1 g/L of Tris base; adjust pH to 6.8 with HCl).
20. Electrophoresis buffer: 25 m*M* Tris, 192 m*M* glycine, pH 8.3.3 g/L of Tris base, 14.4 g/L of glycine; adjust pH to 8.3 if necessary with HCl.
21. 10% Ammonium persulfate in water (made fresh or stored at −20°C in aliquots).
22. *N*,*N*,*N* ,*N* -Tetramethylethylenediamine (TEMED).
23. Vertical slab gel electrophoresis system (minigel or standard size).
24. D.C. Power supply unit (to supply up to 500–1000 V and 200 mA).

25. UV transilluminator (312 nm maximum emission wavelength).
26. Glass UV bandpass filter larger than gel size (type UG-11, or M-UG2).
27. Charge coupled device (CCD) imaging camera fitted with a 450-nm (blue) bandpass filter.

3. Methods

3.1. Tagging Saccharides with a Fluorophore

HS (and heparin) saccharides can be endlabeled by reaction of their reducing aldehyde functional group with a primary amino group of a fluorophore (reductive amination). For sulfated saccharides anthranilic acid (2-AA; ***11***) has been found to be effective for the IGS methodology. 2-AA conjugates display an excitation maxima in the range 300–320 nm, which is ideal for visualization with a commonly available 312-nm UV source (e.g., transilluminators used for visualizing ethidium bromide stained DNA). Emission maxima are typically in the range 410–420 nm (bright violet fluorescence). It has also been found that approx 10-fold more sensitive detection is possible using an alternative fluorophore ANDSA, and this has been demonstrated to be effective for HS sequencing ***(16)***. ANDSA has an excitation maxima of 350 nm and emission maxima of 450 nm. Both approaches described in **Subheadings 3.1.1.** and **3.1.2.** allow rapid labeling and purification of tagged saccharide from free tagging reagent, and give quantitative recoveries and products free of salts that might interfere with subsequent enzymic conditions. For saccharides in the size range hexa to dodecasaccharides, approx 2–3 nmol (approx 2–10 µg) of purified starting material is the minimum required using the 2-AA label and approx 5- to 10-fold less for ANDSA labeling. It should be noted that recently a novel label, BODIPY hydrazide, has been developed for highly sensitive detection of saccharides including those from HS (***17***). This label couples more efficiently to saccharides, has an excitation maxima at 503nm (making it ideal for detection using common 488nm lasers) and an extinction co-efficient of 71,000; this permits very sensitive detection of labelled saccharides (~100fmol on a standard HPLC fluorimeter). In the future it may prove possible to apply this label to highly sensitive IGS sequencing on gels using fluorescent gel scanners or alternatively HPLC or capillary electrophoresis separations with in-line fluorescence detection.

3.1.1. Labeling Saccharides with 2-AA

1. Dry down the purified saccharide (typically 2–10 nmol) in a microcentrifuge tube by centrifugal evaporation.
2. Dissolve directly in 10–25 µL of formamide containing freshly prepared 400 m*M* 2-AA (54.8 mg/mL) and 200 m*M* reductant (sodium cyanoborohydride; 12.6 mg/mL) and incubate at 37°C for 16–24 h in a heating block or oven. **(Caution: The reductant is toxic and should be handled with care.)** The volume used should be sufficient to provide a 500–1000-fold molar excess of 2-AA over saccharide (*see* **Note 1**).

3. Remove free 2-AA, reductant, and formamide from the labeled saccharides by gel filtration chromatography (Sephadex G-25 Superfine). Dilute the sample (maximum 250 µL of reaction mixture) to a total of 1 mL with distilled water (*see* **Note 2**).
4. Load sample onto two 5-mL HiTrap™ Desalting columns (Pharmacia Ltd.) connected in series. Alternatively it is possible to use self-packed columns of other dimensions.
5. Elute with distilled water at a flow rate of 1 mL/min and collect fractions of 0.5 mL. Saccharides consisting of four or more monosaccharide units typically elute in the void volume (approx fractions 7–12). Note that the HiTrap™ columns can be eluted by hand with a syringe without need for a pump.
6. Pool and concentrate these fractions by centrifugal evaporation or freeze drying.

3.1.2. Labeling Saccharides with ANDSA

1. Dry down the purified saccharide (typically 2–10 nmol) in a microcentrifuge tube by centrifugal evaporation.
2. Dissolve directly in 10 µL of formamide.
3. Mix with 15 µL of formamide containing ANDSA at a concentration of 80 mg/mL (approaching saturation) and incubate at 25°C for 16 h.
4. Mix with 10 µL of formamide containing 1 mg of the reductant sodium triacetoxyborohydride (*see* **Note 1**) and incubate for 2 h at 25°C.
5. Remove free ANDSA by gel filtration as described in **Subheading 3.1.1.** for 2-AA.

3.2. Nitrous Acid Treatment of Saccharides

Low pH nitrous acid cleaves HS only at linkages between *N*-sulfated glucosamine and adjacent hexuronic acid residues ***(18,19)***. Under mild controlled conditions nitrous acid cleavage creates a ladder of bands corresponding to the positions of internal *N*-sulfated glucosamine residues in the original intact saccharide ***(11)***. A series of different reaction stop points are pooled, resulting in a partial digest with a range of different fragment sizes.

1. Dry down 1–2 nmol of labeled saccharide by centrifugal evaporation.
2. Redissolve in 80 µL of distilled water and chill on ice.
3. Add 10 µL of 200 m*M* HCl and 10 µL of 20 m*M* sodium nitrite (both prechilled on ice) and incubate on ice.
4. At a series of individual time points (typically 15, 30, 60, 120, and 180 min), remove an aliquot and stop the reaction by raising the pH to approx 5.0 by the addition of 1/5 volume of 200 m*M* sodium acetate buffer, pH 6.0 (*see* **Note 3**).
5. Pool the set of aliquots and either use directly for enzyme digests or desalt as described in

3.3. Exoenzyme Treatment of Saccharides

The approach for treatment of HS samples with exoenzymes is described below. Details of the specificities of the exoenzymes is given in **Table 1**. These enzymes have differing optimal pH and buffer conditions, but in general they

Table 1
Exoenzymes for Sequencing Heparan Sulfate and Heparin

Enzyme*a*	Substrate specificity*b*
Sulfatases	
Iduronate-2-sulfatase	IdoA(2*S*)
Glucosamine-6-sulfatase	GlcNAc(6*S*), GlcNSO3(6*S*)
Sulphamidase (glucosamine *N*-sulfatase)	GlcNSO3
Glucuronate-2-sulfatase	GlcA(2*S*)
Glucosamine-3-sulfatase	GlcNSO3(3*S*)
Glycosidases	
Iduronidase	IdoA
Glucuronidase	GlcA
α-*N*-Acetylglucosaminidase	GlcNAc
Bacterial exoenzymes	
Δ4,5-Glycuronate-2-sulfatase	ΔUA(2*S*)
Δ4,5-Glycuronidase	ΔUA

[a] Enzyme availability: Glucuronidase is widely available commercially as purified or recombinant enzyme. Recombinant iduronate-2-sulfatase, iduronidase, glucosamine-6-sulfatase, sulfamidase, and α-*N*-acetylglucosaminidase were purchased from from the Glyko product range, now supplied by Prozyme (San Leandro, CA; www.prozyme.com). Glucuronate-2-sulfatase and glucosamine-3-sulfatase have only been purified from cell and tissue sources to date. The bacterial exoenzymes are available from Grampian Enzymes, Nisthouse, Harray, Orkney, Scotland; e-mail, grampenz@aol.com.

[b] The specificities are shown as the nonreducing terminal group recognized by the enzymes.

can be used under the single set of conditions given here, which simplifies the multiple enzyme treatments required (*see* **Note 4**).

Sulfatases remove only the sulfate group, whereas the glycosidases cleave the whole nonsulfated monosaccharide.

1. Dissolve the sample (typically 10–200 pmol of saccharide) in 10 µL of H_2O in a microcentrifuge tube.
2. Add 5 µL of exoenzyme buffer (100 m*M* sodium acetate buffer, pH 4.5), 1 µL of 0.5 mg/mL bovine serum albumin, 2 µL of appropriate exoenzyme [0.2–0.5 mU], and distilled water to bring the final volume to 20 µL.
3. Mix the contents well on a vortex mixer, and centrifuge briefly to ensure that the reactants are at the tip of the tube.
4. Incubate the samples at 37°C for 16 h in a heating block or oven.

3.4. Separation of Saccharides by PAGE

PAGE is a high-resolution technique for the separation of HS and heparin saccharides of variable sulfate content and disposition. Its resolution is generally superior to gel filtration or anion-exchange HPLC ***(20,21)***. Improved resolution can be obtained using gradient gels, although these are more difficult to prepare and use routinely. In most cases sufficient resolution can be obtained with isocratic gels (*see* **Note 5**). PAGE provides a simple but powerful approach for separating the saccharide products generated in the sequencing process.

3.4.1. Preparing the PAGE Gel

1. Assemble the gel unit (consisting of glass plates and spacers, etc).
2. Prepare and degas the resolving gel acrylamide solution without ammonium persulfate or TEMED. To make a 30% acrylamide gel solution for a 16 cm • 12 cm • 0.75 mm gel, 16 mL is required. Mix 9.6 mL of T50%–C5% acrylamide stock with 3 mL of 2 *M* Tris, pH 8.8, and 3.4 mL of distilled water.
3. Add 10% ammonium persulfate (30 µL) and TEMED (10 µL) to the gel solution, mix well, and immediately pour into the gel unit.
4. Overlay the unpolymerized gel with resolving gel buffer (375 m*M* Tris-HCl, pH 8.8, diluted from the 2 *M* stock solution) or water-saturated butanol. Polymerization should occur within approx 30–60 min. The gel can then be used immediately or stored at 4°C for 1–2 wk.

3.4.2. Electrophoresis

1. Immediately before electrophoresis, rinse the resolving gel surface with stacking gel buffer (0.125 *M* Tris-HC1 buffer, pH 6.8, diluted from the 1 *M* stock solution).
2. Prepare and degas the stacking gel solution (for 5 mL, mix 0.5 mL of T50%–C5% acrylamide stock with 0.6 mL of 1 *M* Tris, pH 6.8, and 3.9 mL of distilled water).
3. Add 10% ammonium persulfate (10 µL) and TEMED (5 µL). Immediately pour onto the top of the resolving gel and insert the well-forming comb.
4. After polymerization (approx 15 min) remove the comb and rinse the wells thoroughly with electrophoresis buffer.
5. Place the gel unit into the electrophoresis tank and fill the buffer chambers with electrophoresis buffer.
6. Load the oligosaccharide samples (5–20 µL dependent on well capacity, containing approx 10% [v/v] glycerol or sucrose in 125 m*M* Tris-HCl, pH 6.8) carefully into the wells with a microsyringe. Marker samples containing bromophenol blue and phenol red should also be loaded into separate tracks.
7. Run the samples into the stacking gel at 150–200 V (typically 20–30 mA) for 30–60 min, followed by electrophoresis at 300–400 V (typically 20–30 mA and decreasing during run) for approx 5–8 h (for a 16-cm gel). Heat generated during the run should be dissipated using a heat exchanger with circulating tap water, or by running the gel in a cold room or in a refrigerator.
8. Electrophoresis should be terminated before the phenol red marker dye is about 5 cm from the bottom of the gel. (At this point, disaccharides should be 3–4 cm from the bottom of the gel.)

3.5. Gel Imaging

The most effective approach for gel imaging requires a CCD camera that can detect faint fluorescent banding patterns by capturing multiple frames. Systems commonly used for detection of ethidium bromide stained DNA can usually be adapted with appropriate filters as described below (*see* **Note 6**).

1. Place a UV filter (UG-1, UG-11, or MUG-2) onto the transilluminator, and fit a 450-nm blue filter onto the camera lens.
2. Remove the gel carefully from the glass plates after completion of the run and place on the UV transilluminator surface wetted with electrophoresis buffer. Wet the upper surface of the gel to reduce gel drying and curling.
3. Switch on the transilluminator and capture the image using the CCD camera. Exposure times are typically 1–5 s depending on the amount of labeled saccharide (*see* **Note 7**).

3.6. Data Interpretation

The sequence of saccharides can be read directly from the banding pattern by interpreting the band shifts due to removal of specific sulfate or sugar moieties.

Fig. 1 shows an actual example and a schematic representation. First, bands generated by the partial nitrous acid treatment indicate the positions of *N*-sulfated glucosamine residues in the original saccharide (**Fig. 1C**, *track 2*). A "missing" band in the ladder at a particular position indicates the presence of an *N*-acetylated glucosamine residue in the original saccharide at that position (an example of this is shown in **Fig. 3**). Such saccharides can be sequenced by the additional use of the exoenzyme *N*-acetylglucosaminidase, which removes this residue and allows further sequencing of an otherwise "blocked" fragment. Following the nitrous acid treatment, the "ladder" of bands is then subjected to various exoenzyme digestions. The presence of specific sulfate or sugar residues can be deduced from the band shifts that occur (**Fig. 1C**, *tracks 3–5*). **Figure 4** shows an example of a decasaccharide from HS that has been purified by SAX-HPLC and sequenced using IGS ***(11)***.

Although the band shifts are usually downwards (because of to the lower molecular mass and thus higher mobility of the product) it should be noted that occasionally upward shifts occur, probably due to subtle differences in charge/mass ratio (for examples, *see* **Figs. 1B**, **3B**, and **4C**). Note also that minor "ghost" bands sometimes appear after the nitrous acid treatment. They are probably due to loss of an *N*-sulfate group, and normally these do not affect interpretation of the shifts in the major bands ***(11)***.

If the saccharide being sequenced was derived by bacterial lyase treatment, it will have a Δ4,5-unsaturated uronate residue at its nonreducing terminus. If this residue has a 2-*O*-sulfate attached, this can be detected by susceptibility to I2Sase (*see* **Fig. 3B**), but the sugar residue itself is resistant to both Idase and Gase. Its removal is required to confirm whether there is a 6-*O*-sulfate on the adjacent nonreducing end glucosamine (*see* **Figs. 3B** and **4C** for examples). Bacterial enzymes that specifically remove the Δ4,5-unsaturated uronate residues (and the 2-*O*-sulfate groups that may be present on them) are now available

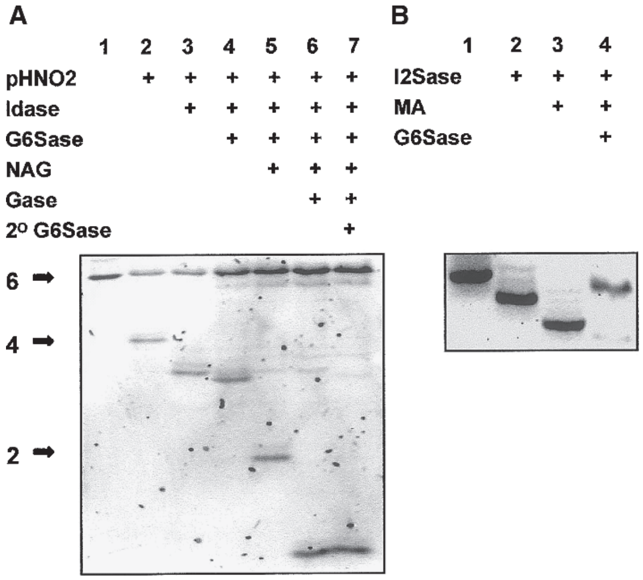

Fig. 3. IGS of a heparin hexasaccharide. A heparin hexasaccharide with the structure ΔHexA(2S)-GlcNSO3(6S)-IdoA-GlcNAc(6S)-GlcA-GlcNSO3(6S) was 2-AA-tagged and subjected to sequencing on a 16-cm 33% gel. (**A**) IGS of hexasaccharide using the combinations of $pHNO_2$ and exoenzyme treatments indicated (*track 1*, untreated, 20 pmol; other tracks correspond to approx 90 pmol per track of starting sample for $pHNO_2$ digest). NAG, *N*-acetylglucosaminidase. (**B**) Determining the sequence of the nonreducing disaccharide unit of the hexasaccharide using the I2Sase, G6Sase, and mercuric acetate (MA) treatments shown (approx 20 pmol per track; *track 1*, untreated). (From ref. 11, Copyright 1999 National Academy of Sciences, USA.)

commercially (*see* **Table 1**). Alternatively, they can be removed chemically with mercuric acetate (*22*; *see* **Figs. 3B** and **4C**).

In addition to the basic sequencing experiment, it is wise to confirm agreement of the data with an independent analysis of the disaccharide composition of the saccharide *(11)*. It can sometimes be difficult to sequence the reducing terminal monosaccharide owing to it being a poor substrate for the exoenzymes. In these cases it has proved more effective to analyze the terminal 2AA-labeled disaccharide unit in comparison to 2AA-labeled disaccharide standards *(11)*. In the future it is anticipated that methods using HPLC or capillary electrophoresis separations and the fluorophore BODIPY hydrazide (*17*) will permit ultra-high sensitivity IGS sequencing of HS saccharides, superceding the standard electrophoretic methods described in this chapter.

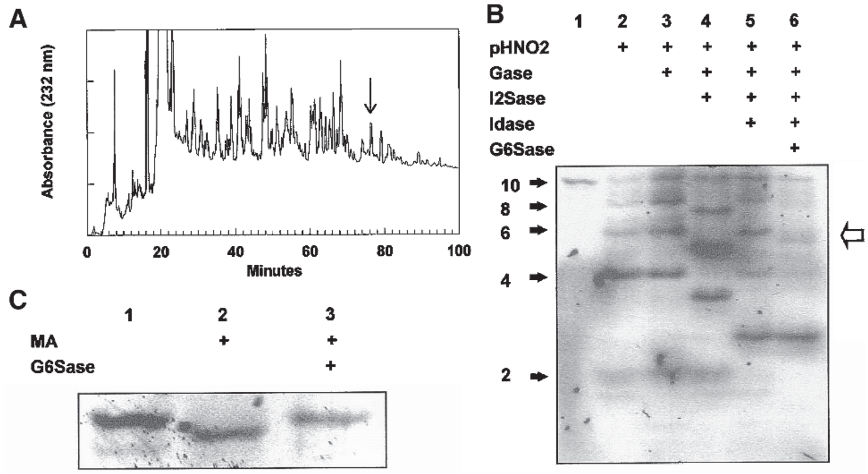

Fig. 4. HPLC purification and IGS of a HS decasaccharide. (**A**) SAX-HPLC of a pool of HS decasaccharides derived by heparitinase treatment of porcine mucosal HS (for details *see* **ref**. 11). The *arrowed peak* was selected for sequencing. (**B**) IGS of the purified HS decasaccharide on a 16-cm 33% gel using the combinations of $pHNO_2$ and exoenzyme treatments indicated (*track 1*, untreated, 20 pmol; other tracks correspond to approx 400 pmol per track of starting sample for $pHNO_2$ digest). (**C**) Determining the sequence of the nonreducing disaccharide unit of the HS decasaccharide using the mercuric acetate (MA) and G6Sase treatments shown (approx 40 pmol per track; *track 1*, untreated). (From ref. 11, Copyright 1999 National Academy of Sciences, USA.)

4. Notes

1. Using large excesses of reagent as described, saccharides derived from HS and heparin by bacterial lyase scission generally couple with 2-AA with efficiencies in the range of 60–70%. Note that saccharides derived from HS and heparin by low pH nitrous acid scissioning (i.e., having an anhydromannose residue at their reducing ends) label more efficiently (approx 70–80% coupling efficiency). Labeling with ANDSA achieves similar coupling efficiencies, and the alternative reducing agent, sodium triacetoxyborohydride, is less toxic than sodium cyanoborohydride.
2. Unwanted reactants and solvent can also be removed from labeled saccharides by methods such as dialysis but the rapid gel filtration chromatography step described above using the HiTrap desalting columns is convenient and usually allows good recoveries of loaded sample (typically 70–80%).
3. It is best to perform some trial incubations to test for optimal time points needed to generate a balanced mix of all fragments in the partial nitrous

acid digestion. With longer saccharides (octasaccharides and larger) it is observed that the largest products are generated quickly and thus a bias toward shorter incubation times is required as saccharide length increases ***(16)***.

4. The enzyme conditions described should provide for complete digestion of all susceptible residues. This is important to the sequencing process, as incomplete digestion would create a more complex banding pattern and would give a false indication of sequence heterogeneity. It is useful to run parallel controls with standard saccharides to enable monitoring of reaction conditions. Where combinations of exoenzymes are required, these can be incubated simultaneously with the sample. If required, the activity of one enzyme can be destroyed prior to a secondary digestion with a different enzyme by heating the sample at 100°C for 2–5 min.
5. Adequate separations, particularly over limited size ranges of saccharides, can be obtained using single concentration gels, typically in the range 25–35% acrylamide. Improvements in resolution can be made by using longer gel sizes. Different voltage conditions (usually in the range 200–600 V) and running times are required for different gel formats, and should be established by trial and error with the particular samples being analyzed. Gels up to 24 cm in length can usually be run in 5–8 h using high voltages, whereas for longer gels it is often convenient to use lower voltage conditions and overnight runs. Minigels can also be used effectively for separation of small HS–heparin saccharides up to octasaccharides in size (*see* **Fig. 1**). Note that it is also possible to run Tris-acetate gels with a Tris-MES electrophoresis buffer (*see* **Fig. 1**; ***11***).
6. Because the emission wavelength of 2-AA tagged saccharides is 410–420 nm, there is a need to filter out background visible wavelength light from the UV lamps. This can be done effectively with special glass filters that permit transmission of UV light but do not allow light of wavelengths >400 nm to pass. A blue bandpass filter on the camera also improves sensitivity. Suitable filters are available from HV Skan (Stratford Road, Solihull, UK; Tel: 0121 733 3003) or UVItec Ltd. (St. John's Innovation Centre, Cowley Road, Cambridge, UK; www.uvitec.demon.co.uk).
7. Required exposure times are strongly dependent on sample loading and the level of detection required. Over-long exposures will result in excessive background signal. Note that negative images are usually better for band identification (*see* figures). Under the conditions described the limit of sensitivity is approx 10–20 pmol per band of original starting material for 2-AA (*see* **Fig. 1**) and 2–5 pmol per band for ANDSA.

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Analysis of Glycoprotein Heterogeneity by Capillary Electrophoresis and Mass Spectrometry

Andrew D. Hooker and David C. James

1. Introduction

The drive toward protein-based therapeutic agents requires both product quality and consistency to be maintained throughout the development and implementation of a production process. Differences in host cell type, the physio-logical status of the cell, and protein structural constraints are known to result in variations in posttranslational modifications that can affect the bioactivity, receptor binding, susceptibility to proteolysis, immunogenicity, and clearance rate of a therapeutic recombinant protein in vivo ***(1)***. Glycosylation is the most extensive source of protein heterogeneity, and many recent developments in analytical biotechnology have enhanced our ability to monitor and structurally define changes in oligosaccharides associated with recombinant proteins.

Variable occupancy of potential glycosylation sites may result in extensive glycosylation macroheterogeneity in addition to the considerable diversity of carbohydrate structures that can occur at individual glycosylation sites, often referred to as glycosylation microheterogeneity. Variation within a heterogeneous population of glycoforms may lead to functional consequences for the glycoprotein product. Therefore, regulatory authorities such as the US Food and Drug Administration (FDA) and the Committee for Proprietary Medical Productions demand increasingly sophisticated analysis for biologics produced by the biotechnology and pharmaceutical industries ***(2)***. The FDA has described a “well-characterized biologic” as “a chemical entity whose identity, purity, impurities, potency and quantity can be determined and controlled.”

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The glycosylation of a recombinant protein product can be examined by:

1. Analysis of glycans released by chemical or enzymatic means.
2. Site-specific analysis of glycans associated with glycopeptide fragments following proteolysis of the intact glycoprotein.
3. Direct analysis of the whole glycoprotein.

A number of techniques are currently available to provide rapid and detailed analysis of glycan heterogeneity:

1. High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).
2. Enzymatic analysis methods such as the reagent array analysis method (RAAM; ***3,4***).
3. High-performance capillary electrophoresis (HPCE).
4. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).
5. Electrospray ionization mass spectrometry (ESI-MS).

In particular, novel mass spectrometric strategies continue to rapidly advance the frontiers of biomolecular analysis, with technical innovations and methodologies yielding improvements in sensitivity, mass accuracy, and resolution.

1.1. High-Performance Capillary Electrophoresis

Capillary electrophoresis has been employed in various modes in the high-resolution separation and detection of glycoprotein glycoforms, glycoconjugates, glycopeptides, and oligosaccharides, even though carbohydrate molecules do not absorb or fluorese and are not readily ionized ***(5–8)***. A number of approaches have been employed to render carbohydrates more amenable to analysis which include *in situ* complex formation with ions such as borate and metal cations ***(9)*** and the addition of ultraviolet (UV)-absorbing or fluorescent tags to functional groups ***(10)***.

1.2. MALDI-MS

MALDI-MS has been extensively used to determine the mass of proteins and polypeptides, confirm protein primary structure, and to characterize posttranslational modifications. MALDI-MS generally employs simple time-of-flight analysis of biopolymers that are co-crystallized with a molar excess of a low molecular weight, strongly UV absorbing matrix, such as 2,5-dihydroxybenzoic acid, on a metal sample disk. Both the biopolymer and matrix ions are desorbed by pulses of a UV laser. Following a linear flight path the molecular ions are detected, the time between the initial laser pulse and ion detection being directly proportional to the square root of the molecular ion mass/charge (*m/z*) ratio. For maximum mass accuracy, internal and external protein or peptide calibrants of known molecular mass are required. In addition to this "linear" mode, many instruments offer a "reflectron" mode that effectively lengthens the flight path by redirecting the ions toward an additional ion detector that may enhance resolution, at the expense of decreased sensitivity. MALDI-MS is tolerant to low (micromolar) salt concentrations, can determine the molecular weight of biomolecules in excess of 200 kDa with a mass accuracy of ±0.1%, and is capable of analyzing heterogeneous samples with picomole to femtomole sensitivity. These properties combined with its rapid analysis time and ease of use for the nonspecialist have made it an attractive technique for the analysis of glycoproteins, glycopeptides, and oligosaccharides.

1.3. Electrospray Ionization Mass Spectrometry

ESI-MS is another mild ionization method, where the covalent bonding of the biopolymer is maintained and is typically used in combination with a single or triple quadrupole. This technique is capable of determining the molecular weight of biopolymers up to 100 kDa with a greater mass accuracy (±0.01%) and resolution (±2000) than MALDI-MS. Multiply charged molecular ions are generated by the ionization of biopolymers in volatile solvents, the resulting spectrum being convoluted to produce noncharged peaks.

ESI-MS has been extensively used for the direct mass analysis of glycopeptides and glycoproteins and is often interfaced with liquid chromatography ***(11–13)***, but has found limited application for the direct analysis of oligosaccharides ***(14)***. ESI-MS is better suited to the analysis of whole glycoprotein populations than MALDI-MS, its superior resolution permitting the identification of individual glycoforms ***(15, 16)***.

2. Materials

1. P/ACE 2100 HPCE System (Beckman Instruments Ltd., High Wycombe, UK).
2. Phosphoric acid (Sigma Chemical Co., Poole, UK).
3. Boric acid (Sigma).
4. Trypsin, sequencing grade (Boehringer Mannheim, UK, Lewes, UK).
5. Waters 626 Millenium HPLC System (Millipore Ltd., Watford, UK).
6. Vydac 218TP52 reverse-phase column: C18, 2.1 × 250 mm (Hichrom Ltd., Reading, UK).
7. HPLC grade water-acetonitrile (Fischer Scientific, Loughborough, UK).
8. α-Cyano-4-hydroxy cinnaminic acid (Aldrich Chemical Co., Gillingham, UK).
9. VG Tof Spec Mass Spectrometer (Fisons Instruments, Manchester, UK).
10. Vasoactive intestinal peptide—fragment 1–12 (Sigma).
11. Peptide-*N*-glycosidase F and glycosidases (Glyko Inc., Upper Heyford, UK).
12. 2,5-Dihydroxybenzoic acid (Aldrich).
13. 2,4,6-Trihydroxyacetophenone (Aldrich).
14. Ammonium citrate (Sigma).
15. VG Quattro II triple quadrupole mass spectrometer (VG Organic, Altrincham, UK).
16. Horse heart myoglobin (Sigma).

3. Methods

This chapter describes some of the recent technological advances in the analysis of posttranslational modifications made to recombinant proteins and focuses on the application of HPCE, MALDI-MS, and ESI-MS to the monitoring of glycosylation heterogeneity. These techniques are illustrated by describing their application to the analysis of recombinant human γ-interferon (IFN-γ), a well-characterized model glycoprotein that has *N*-linked glycans at Asn_{25} and at the variably occupied site, Asn_{97} ***(17)***.

3.1. Glycosylation Analysis by HPCE

Micellar electrokinetic capillary chromatography (MECC) can be used to rapidly "fingerprint" glycoforms of recombinant human IFN-γ produced by Chinese Hamster Ovary (CHO) cells ***(8)*** and to quantitate variable-site occupancy (macroheterogeneity; *see* **Fig. 1**). This approach allows glycoforms to be rapidly resolved and quantified without the need for oligosaccharide release, derivatization or labeling.

1. Separations are performed with a P/ACE 2100 capillary electrophoresis system using a capillary cartridge containing a 50 mm internal diameter (i.d.) × 57 cm length of underivatized fused silica capillary.
2. Buffer solutions are prepared from phosphoric and boric acids using NaOH to adjust the pH.
3. Capillaries are prepared for use by rinsing with 0.1 *M* NaOH for 10 min, water for 5 min, 0.1 *M* borate, pH 8.5, for 1 h, then 0.1 *M* NaOH and water for 10 min,

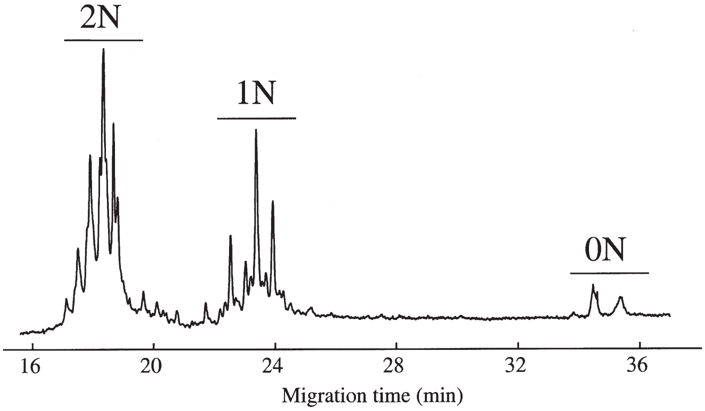


Fig. 1. Whole recombinant human IFN-γ analyzed by capillary electrophoresis. Recombinant human IFN-γ glycoforms were "fingerprinted" by micellar electrokinetic capillary chromatography. Peak groups represent IFN-γ variants with both Asn sites occupied (2N), one site occupied (1N), or no sites occupied (0N).

respectively. Prior to use, capillaries are equilibrated with electrophoresis buffer (400 m*M* borate + 100 m*M* sodium dodecyl sulfate [SDS], pH 8.5) for 1 h.

4. Voltages are applied over a 0.2-min linear ramping period at a detection wavelength of 200 nm and operating temperature of 25°C. Recombinant human IFN-γ (1 mg/mL in 50 m*M* borate, 50 m*M* SDS, pH 8.5) is injected for 5 s prior to electrophoresis at 22 kV. Between each separation, the capillary is rinsed with 0.1 *M* NaOH, water, and electrophoresis buffer for 5 min, respectively (*see* **Note 1**).

3.2. Glycosylation Analysis by MALDI-MS

There are only a few reports of the analysis of whole glycoproteins due to the limited resolution of this technique ***(18)***. As a result, analysis of intact glycoproteins is generally limited to those proteins that contain one glycosylation site and are < 15–20 kDa ***(15)***.

MALDI-MS has proved more useful for the identification and characterization of glycopeptides following their separation and purification by reverse-phase HPLC ***(19)***. The advantage of this approach over other methods is that site-specific glycosylation data can be obtained (***20,21***; *see* **Note 2**). This approach has been successfully used to determine the differences in *N*-linked glycosylation for the Asn_{25} and Asn_{97} sites of recombinant human IFN-γ when produced in different expression systems ***(22)***, to monitor changes during batch culture ***(23)***, and to monitor intracellular populations (***24***; *see* **Fig. 2**).

1. Purified IFN-γ samples are digested with trypsin (1.5 μγ; 50 μg) for 24 h at 30°C.
2. The glycopeptides containing the *N*-glycan populations are isolated following their separation by reverse-phase HPLC. Samples are applied in 0.06% (v/v) trifluoroacetic acid (TFA) and the peptides separated with a linear gradient (0–70%) of 80% (v/v) aqueous CH_3CN with 0.052% TFA over 100 min at a flow rate of 0.1 ml/min. Peptide peaks are detected at a wavelength of 210 nm and collected individually.
3. The glycopeptides are reduced to the aqueous phase in a Speed Vac concentrator, lyophilized overnight, and stored at –20°C.
4. A 0.5-μL aliquot of the digest samples is mixed with 0.5 μL of a saturated solution of α-cyano-4-hydroxy cinnaminic acid in 60% (v/v) aqueous CH_3CN and allowed to co-crystallize on stainless steel sample discs.
5. MALDI-MS is performed with a N_2 laser at 337 nm. Desorbed positive ions are detected after a linear flight path by a microchannel plate detector and the digitalized output signal adjusted to obtain an optimum output signal-to-noise ratio from 20 averaged laser pulses. Mass spectra are calibrated with an external standard, vasoactive intestinal peptide with an average molecular mass of 1425.5.
6. Digestion of glycopeptides with peptide-*N*-glycosidase F (PNGaseF) prior to analysis by mass spectrometry confirms the mass of the core peptide. For this determination, 0.5 μL of sample and 0.5 μL of PNGaseF are incubated at 30°C for 24 h and 0.5-μL aliquots are removed for MALDI-MS analysis.
7. Simultaneous digestion of purified glycopeptides with linkage-specific exoglycosidase arrays for the sequential removal of oligosaccharides permit the sequen-cing of

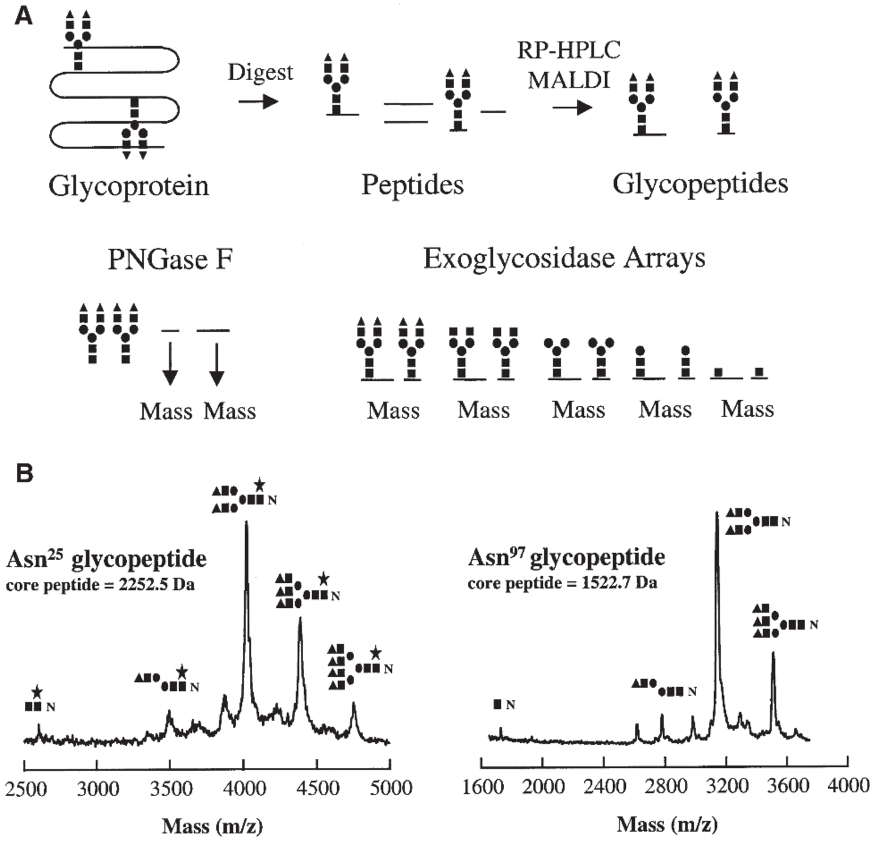


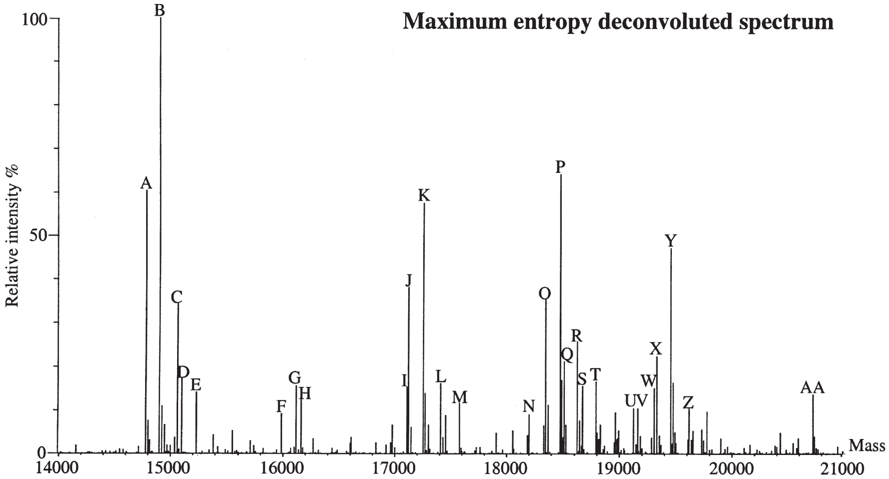
Fig. 2. Site-specific *N*-glycosylation of recombinant human IFN-γ examined by MALDI-MS analyses of glycopeptides. **(A)** The complete analytical protocol. The masses of individual *N*-glycans at a single glycosylation site were calculated by subtracting the known mass of the core peptide moiety from each component in a glycopeptide spectrum. **(B)** An *N*-glycan structure was then tentatively assigned, based on mass criteria alone. As ionization is entirely dependent on the core peptide moiety after desialylation, individual *N*-glycan structures can be quantified. Monosaccharide structures are schematically represented as: (▲), galactose (162.14); (■), *N*-acetylglucosamine (203.20 Da); (●), mannose (162.14 Da); and (★), fucose (146.14 Da).

N-glycans at individual glycosylation sites by MALDI-MS ***(21–24)***. Sample (0.5 µL) and glycosidase (0.5 µL), or a combination of glycosidases, are incubated at 30°C for 24 h and 0.5-µL aliquots are removed for MALDI-MS analysis (*see* **Note 3**).

3.3. Glycosylation Analysis by ESI-MS

ESI-MS has been used to aid the analysis of glycosylation macro- and microheterogeneity and proteolytic cleavage of the C-terminal in conjunction with information obtained by HPLC and MALDI-MS of released oligosaccharides (*25*; *see* **Note 4**).

1. Spectra are obtained with a VG Quattro II triple quadrupole mass spectrometer having a mass range for singly charged ions of 4000 Da **(Fig. 3)**.
2. Lyophilized proteins are dissolved in 50% aqueous acetonitrile, 0.2% formic acid to a concentration of 0.1 µg/µL and introduced into the electrospray source at 4 µL/min. The mass-to-charge (*m/z*) range of 600–1800 Da are scanned at 10 s/scan and data are summed for 3–10 min, depending on the intensity and complexity of the spectra.



Component	Observed mass (Da)	Correlating mass (Da)	Polypeptide length	Asn sites occupied	Monosaccharide composition
A	14772.5	14771.8	1-127	0	
B	14900.4	14900.0	1-128	0	
C	15056.8	15056.1	1-129	0	
D	15096.9	NA			
E	15224.3	NA			
F	15988.3	15988.9	1-127	1	H5 N2
G	16116.5	16117.1	1-128	1	H5 N2
H	16160.4	16160.5	1-138	0	
I	17107.0	17106.0	1-128	1	H5 N4 S2
J	17124.4	17123.9	1-127	1	H5 N4 S2 D1
K	17253.0	17252.1	1-128	1	H5 N4 S2 D1
L	17409.1	17408.3	1-129	1	H5 N4 S2 D1
M	17576.0	NA			
N	18195.6	18194.9	1-127	2	H10 N6 S2
O	18341.0	18341.0	1-127	2	H10 N6 S2 D1
P	18470.0	18469.2	1-128	2	H10 N6 S2 D1
Q	18511.9	18512.6	1-138	1	H5 N4 S2 D1
R	18627.2	18625.4	1-129	2	H10 N6 S2 D1
S	18668.8	18668.8	1-139	1	H5 N4 S2 D1
T	18793.8	NA			
U	19124.3	19125.0	1-133	2	H10 N6 S2 D1
V	19165.5	19166.9	1-128	2	H10 N8 S3 D1
W	19312.8	19312.0	1-128	2	H10 N8 S4
		19313.0*	1-128	2	H10 N8 S3 D2
X	19329.2	19329.9	1-127	2	H10 N8 S4 D1
Y	19458.6	19458.1	1-128	2	H10 N8 S4 D1
Z	19614.7	19614.3	1-129	2	H10 N8 S4 D1
AA	20719.0	20718.6	1-138	2	H10 N8 S4 D1

Fig. 3. Heterogeneous glycoprotein populations directly analyzed by ESI-MS. This technique provides highly resolved mass analyses with a mass accuracy of 0.01% (1 Dalton/10 kDa). In this example, individual transgenic mouse derived recombinant human IFN-γ components were assigned a C-terminal polypeptide cleavage site and an overall monosaccharide composition.

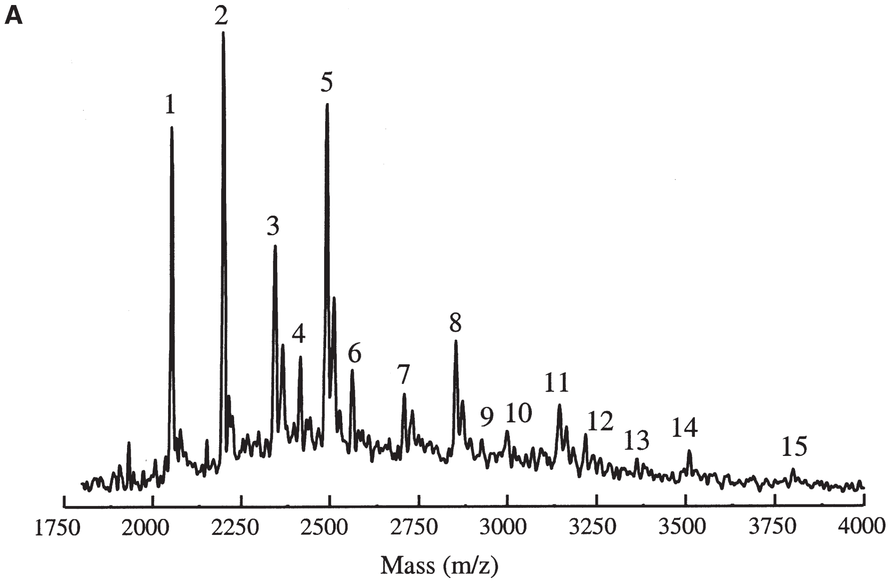
During each scan, the sample orifice-to-skimmer potential (cone voltage) are scanned from 30 V at *m/z* 600 to 75 V at *m/z* 1800. The capillary voltage is set to 3.5 kV.

3. Mass scale calibration employ the multiply charged ion series from a separate introduction of horse heart myoglobin (average molecular mass of 16,951.49). Molecular weights are based on the following atomic weights of the elements: C = 12.011, H = 1.00794, N = 14.00674, O = 15.9994, and S = 32.066 (*see* **Note 5**).
4. Background subtracted *m/z* data are processed by software employing a maximum-entropy (MaxEnt) based analysis to produce zero-charge protein molecular weight information with optimum signal-to-noise ratio, resolution, and mass accuracy.

4. Notes

1. Attempts to separate IFN-γ with borate alone, as used by Landers et al. ***(26)*** for the separation of ovalbumin glycoforms ***(26)***, were unsuccessful, because SDS is required to disrupt the hydrogen-bonded dimers. Application of this technique to ribonuclease B and fetuin met with variable success. The glycoprotein microheterogeneity of a monoclonal antibody with a single glycosylation site has been mapped using a borate buffer at high pH; the glycans were enzymatically or chemically cleaved and the resulting profile used for testing batch-to-batch consistency in conjunction with MALDI-MS analysis ***(27)***.
2. MALDI-MS of free *N*-linked oligosaccharides, following chemical release with hydrazinolysis or enzymatic release with an endoglycosidase such as PNGaseF, is also popular as it requires no prior structural knowledge of the glycoprotein of interest and is ideal for the analysis of underivatized populations of oligosaccharides. However, there is a loss of glycosylation site-specific data. Enzymatic release of oligosaccharides is preferred where an intact deglycosylated protein product is required, as *N*-glycan release by hydrazinolysis may result in peptide bond cleavage and the oligosaccharide product requires reacetylation. A drawback to the enzymatic release of oligosaccharides is that the presence of SDS is often required to denature the glycoprotein and has to be removed prior to MALDI-MS analysis. MALDI-MS has also been used for the analysis of IFN-γ glycoforms separated by SDS-polyacrylamide gel electrophoresis (PAGE) ***(28)***.
3. Until recently, only desialylated oligosaccharides could be analyzed successfully by MALDI-MS using 2,5-dihydroxybenzoic acid as matrix, as negatively charged sialic acids interfere with the efficiency of ionization using this procedure ***(29,30)***. However, advances in matrix mixtures and sample preparation schemes now promise to further improve analytical protocols. For example, sialylated oligosaccharides have recently been shown to ionize effectively, with picomole to femtomole sensitivity, as deprotonated molecular ions using 2,4,6-trihydroxyacetophenone as matrix in the presence of ammonium citrate ***(31)***. Further improvements in sensitivity and resolution may be obtained by derivatization of oligosaccharides with

fluorophores such as 2-aminoacridome (AMAC) or 2-aminobenzamidine (AB; **Fig. 4**). Integration of MALDI-MS peak areas obtained on analysis of sialylated glycans from IFN-γ provided quantitative information that compared favorably with analysis of the derivatized sialylated glycans by ion-exchange HPLC.



B

Component	Observed Mass (Da)	Correlating Mass (Da)	Monosaccharide Composition
1	2052	2052.7	H5N4D0S1
2	2198	2198.8	H5N4D1S1
3	2343	2344.0	H5N4D0S2
4	2416	2418.1	H6N5D0S1
5	2490	2490.1	H5N4D1S2
6	2563	2564.2	H6N5D1S1
7	2709	2709.3	H6N5D0S2
8	2855	2855.5	H6N5D1S2
9	2928	2929.5	H7N6D1S1
10	2998	3000.6	H6N5D0S3
11	3146	3146.7	H6N5D1S3
12	3219	3220.8	H7N6D1S2
13	3364	3366.0	H7N6D0S3
14	3511	3512.0	H7N6D1S3
15	3801	3803.3	H7N6D1S4

Fig. 4. Sialylated *N*-glycans associated with recombinant human IFN-γ released with PNGaseF, labeled with 2-aminobenzamide, and analyzed by MALDI-MS using 2,4,6-trihydroxyacetophenone as matrix (**A**). The masses of individual *N*-glycans are used to assign an overall monosaccharide composition, including degree of sialylation (**B**). H, hexose; N, *N*-acetylhexosamine; D, deoxyhexose; and S, *N*-acetylneuraminic acid.

4. Glycopeptides may be directly analyzed by ESI-MS ***(32,33)***, and the oligosaccharides sequenced following digestion with combinations of exoglycosidases ***(34)*** or glycoforms separated by liquid chromatography prior to analysis ***(35)***. Possibly the most powerful application of this technique has resulted from its interfacing with liquid chromatography which permits the separation and on-line identification of glycoproteins from protein digests ***(36)***. Although ESI-MS may be interfaced with capillary electrophoresis, the separation of glycoproteins and glycopeptides under acidic conditions makes the analysis of sialylated oligosaccharides unsuitable.
5. ESI-MS is not ideally suited for the analysis of neutral and anionic oligosaccharides that have been chemically or enzymatically released from a glycoprotein of interest as they do not readily form multiply charged ions. However, the characterization of methylated derivatives of oligosaccharides from recombinant erythropoietin by ESI-MS has been reported ***(37)***.

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Affinity Chromatography of Oligosaccharides and Glycopeptides with Immobilized Lectins

Kazuo Yamamoto

1. Introduction

Sugar moieties on the cell surface play one of the most important roles in cellular recognition. In order to elucidate the molecular mechanisms of these cellular phenomena, assessment of the structure of sugar chains is indispensable. However, it is difficult to elucidate the structures of cell-surface oligosaccharides because of two technical problems. The first is the difficulty in fractionating various oligosaccharides that are heterogeneous in the number, type, and substitution patterns of the outer sugar branches. The second problem is that only very limited amounts of material may be available, making it difficult to perform detailed structural studies.

Lectins are proteins with sugar-binding activity. Each lectin binds to a specific sugar sequence in oligosaccharides and glycopeptides. Therefore, lectins are very useful tools for overcoming the problems described above. Recently, many attempts have been made to fractionate oligosaccharides and glycopeptides on immobilized-lectin columns. The use of a series of immobilized-lectin columns, with sugar-binding specificities that have been precisely elucidated, has enabled us to fractionate very small amounts (~10 ng) of oligosaccharides or glycopeptides into structurally distinct groups. Here, we summarize our serial lectin-Sepharose affinity-chromatographic techniques for the rapid, sensitive, and specific fractionation and analysis of asparagine-linked oligosaccharides of glycoproteins.

The structures of asparagine-linked oligosaccharides fall into three main categories, termed high mannose-type, complex-type, and hybrid-type oligosaccharides ***(1).*** They share the common core structure, Manα1-3(Manα1-6)

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Manβ1-4GlcNAcβ1-4GlcNAc-Asn, but differ in their outer branches **(Fig. 1).** High mannose-type oligosaccharides have two to six α-mannose residues added to the core structure. Typical complex-type oligosaccharides contain two to four outer branches with a sialyllactosamine sequence. Hybrid-type structures have the features of both high mannose-type and complex-type oligosaccharides and most contain a bisecting *N*-acetylglucosamine, which has a β1-4 linkage to the β-linked mannose residue of the core structure. A novel type of carbohydrate chain, called the poly-*N*-acetyllactosamine-type oligosaccharide, also has been described ***(2–5).*** Its outer branches have a characteristic structure composed of *N*-acetyllactosamine repeating units. Although it may be classified as a complex-type oligosaccharide, it is antigenically and functionally distinct from standard complex-type sugar chains ***(4).*** Some poly-*N*-acetyllactosamine-type oligosaccharides have branched sequences containing Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Gal units ***(2, 3),*** which is the determinant of the I-antigen. Another novel complex-type sugar chain, having GalNAcβ1-4GlcNAc groups in its outer chain moieties, has been found ***(6, 7)*** and GalNAc residues are sometimes sulfated

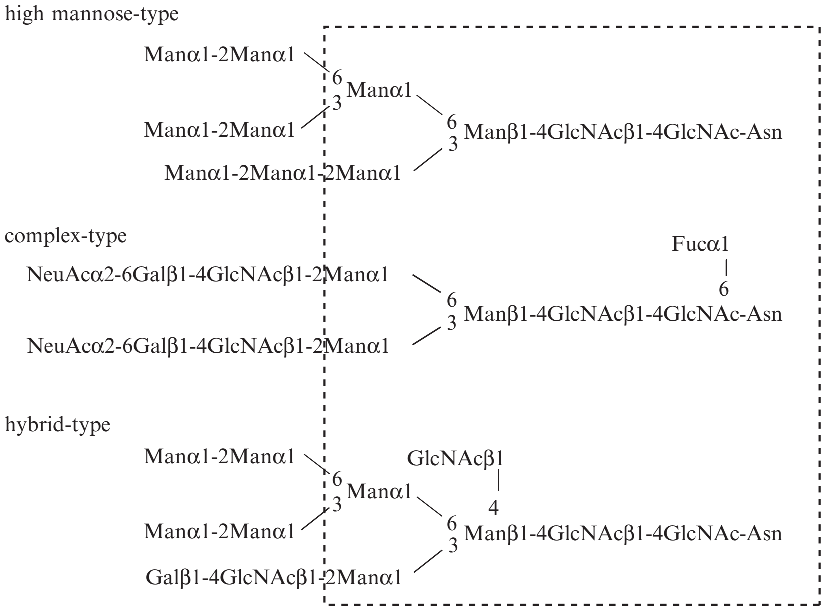

high mannose-type
Manα1-2Manα1
6
3
Manα1
Manα1-2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
Manα1-2Manα1-2Manα1
complex-type
Fucα1
6
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1
hybrid-type
Manα1-2Manα1
GlcNAcβ1
6
3
Manα1
4
Manα1-2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
Galβ1-4GlcNAcβ1-2Manα1

Fig. 1. Structures of major types of asparagine-linked oligosaccharides. The boxed area encloses the core structure common to all asparagine-linked oligosaccharides.

at the C-4 position or sialylated at the C-6 position. Complex-type sugar chains with non-reducing Galα1-3(4)Gal structures have also been reported ***(8).***

Glycopeptides or oligosaccharides can be prepared from glycoproteins by enzymatic digestions or chemical methods, as discussed in subsequent chapters in this book. The most widely used means for preparing glycopeptides is to completely digest the material with pronase. Oligosaccharides can be prepared from glycoproteins or glycopeptides by treating samples with anhydrous hydrazine ***(9)*** or endoglycosidases. Since the released oligosaccharides retain their reducing termini, they can be radiolabeled by reduction with NaB^3H_4 ***(10)*** or fluorescently labeled by 2-aminopyridine ***(11).*** Primary amino groups of the peptide backbone of glycopeptides are labeled by acetylation with [^{3}H]- or [^{14}C]-acetic anhydride (12). Before employing columns of immobilized lectins for analyses, oligosaccharides or glycopeptides should be separated on a column of QAE- or DEAE-cellulose, based on the anionic charge derived from sialic acid, phosphate, or sulfate residues. Separated acidic oligosaccharides should be converted to neutral oligosaccharides to simplify the subsequent separations. For simplicity, the oligosaccharides discussed here do not contain sialic acid, phosphate, or sulfate residues, although these acidic residues, especially sialic acid residues, are found in many oligosaccharides. In most cases, the influence of these residues on the interaction of oligosaccharides with immobilized lectins is weak, but where documentation of the effects of these residues is available, it will be mentioned in the appropriate sections. Here we describe the general procedure for serial lectin affinity chromatography of glycopeptides and oligosaccharides using several well-defined immobilized lectins.

2. Materials

1. Mono Q HR5/50 GL, DEAE-Sepharose, Sephadex G-25 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).
2. High performance liquid chromatograph, two pumps, with detector capable of monitoring ultraviolet absorbance at 220 nm.
3. Neuraminidase: 1 unit/ml of neuraminidase from *Streptococcus sp*. (Seikagaku Kogyo, Tokyo, Japan) in 50 m*M* acetate buffer, pH 6.5.
4. Dowex 50W-X8 (50–100 mesh, H^+ form).
5. Bio-Gel P-4 minus 400 mesh (Bio-Rad, Richmond, CA).
6. HPLC mobile phase for Mono Q: A, 2 m*M* Tris-HCl, pH 7.4; B, 2 m*M* Tris-HCl, pH 7.4, 0.5 *M* NaCl.
7. HPLC mobile phase for Bio-Gel P-4: distilled water.
8. HPLC standard for Bio-Gel P-4: partial hydrolysate of chitin, prepared according to Rupley (13); 10 µg mixed with 50 µl distilled water. Store frozen.
9. Concanavalin A, *Ricinus communis* lectin, wheat germ lectin, *Datura stramonium* lectin, *Maackia amurensis* leukoagglutinin, *Wistaria floribunda* lectin, *Allomyrina dichotoma* lectin, *Amaranthus caudatus* lectin, *Griffonia (Bandeiraea) simplicifolia*

isolectin B_4 (EY Laboratories, San Mateo, CA), *Phaseolus vulgaris* erythroagglutinin, *P. vulgaris* leukoagglutinin (Seikagaku Kogyo). Immobilized lectins are prepared at a concentration of 1–5 mg lectin/ml of gel (*see* **Notes 1** and **2**). The following may be obtained commercially as immobilized lectins: *Galanthus nivalis* lectin, *Lens culinaris* lectin, *Pisum sativum* lectin, *Vicia fava* lectin, pokeweed mitogen, *Sambucus nigra L.* lectin (e.g., GE Healthcare Bio-Sciences AB, EY Laboratories, Bio-Rad, Seikagaku Kogyo).

10. ^{3}H-NaB^{3}H$_4$: 3.7 × 10^9 Bq of ^{3}H-NaBH$_4$ (sp. 1.9–5.6 × 10^{11} Bq/mmol; PerkinElmer, Boston, MA) mixed with 2 ml 10 m*M* NaOH; store at −80°C.
11. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 7.4, 0.15 *M* NaCl.
12. Lectin column buffer: 10 m*M* Tris-HCl, pH 7.4, 0.15 *M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$ (*see* **Note 3**).
13. *N*-acetylgalactosamine (Sigma, St Louis, MO): 100 m*M* in lectin column buffer; store refrigerated.
14. Methyl-α-mannoside (Sigma): 100 m*M* in TBS; store refrigerated.
15. Methyl-α-glucoside (Sigma): 10 m*M* in TBS; store refrigerated.
16. Lactose (Sigma): 50 m*M* in TBS; store refrigerated.
17. *N*-acetylglucosamine (Sigma): 200 m*M* in TBS; store refrigerated.

3. Methods

3.1. Separation of Acidic Sugar Chains on Mono Q or DEAE-Sepharose and Removal of Sialic Acids.

3.1.1. Ion Exchange Chromatography

1. Equilibrate the Mono Q or DEAE-Sepharose column with 2 m*M* Tris-HCl, pH 7.4, at a flow rate of 1 ml/min at room temperature.
2. Dissolve the oligosaccharides or glycopeptides in 0.1 ml of 2 m*M* Tris-HCl, pH 7.4, and apply to the column.
3. Elute with 2 m*M* Tris-HCl, pH 7.4, for 10 min, followed by a linear gradient (0-20%) of 2 m*M* Tris-HCl, pH 7.4, 0.5 *M* NaCl for 60 min, at a flow rate of 1 ml/min.
4. Neutral oligosaccharides are recovered in the pass-through fraction. Acidic monosialo-, disialo-, trisialo-, and tetrasialooligosaccharides are eluted successively by the linear gradient of NaCl.

3.1.2. Removal of Sialic Acid Residues

1. To 10–100 µg oligosaccharides, free of buffers and salts, add 100 µl of neuraminidase buffer and 100 µl of neuraminidase and incubate at 37°C for 18 h.
2. Heat-inactivate the neuraminidase by immersion in a boiling-water bath for 3 min.
3. Apply to the Dowex 50W-X8 column (0.6 cm id × 2.5 cm), wash the column with 1 ml of distilled water, and concentrate the eluate under vacuum.

Alternatively, add 500 µl of 0.1 *M* HCl to 10–100 µg oligosaccharides, heat at 80°C for 30 min, and dry the sample using an evaporator.

3.2. Separation of Poly-*N*-Acetyllactosamine-Type Sugar Chains from Other Types of Sugar Chains

Poly-*N*-acetyllactosamine-type sugar chains vary as to the number of *N*-acetyllactosamine repeating units and their branching modes. Thus, the structural characterization of poly-*N*-acetyllactosamine-type sugar chains has been quite difficult ***(14).*** This type of sugar chain has a higher molecular weight than high mannose-type, complex-type, or hybrid-type chains. Poly-*N*-acetyllactosamine-type sugar chains with molecular masses of more than 4,000 are excluded in the Bio-Gel P4 column chromatography ***(2, 15, 16)*** and thus are easily separated from other types.

1. Equilibrate two coupled Bio-Gel P-4 columns (0.8 cm id × 50 cm) in water at 55°C using a water jacket.
2. Elute the oligosaccharides at a flow rate of 0.3 ml/min and collect 0.5 ml fractions. Monitor absorbance at 220 nm.
3. Collect poly-*N*-acetyllactosamine-type oligosaccharides that elute in the void volume of the column. Other types of oligosaccharides are subjected to subsequent separations **(Subheadings 3.3–3.6),** as illustrated in **Fig. 2.** The specificities of the lectins are summarized in **Fig. 3** and **Table 1.**

3.3. Separation of Complex-Type Sugar Chains Containing GalNAc 1-4GlcNAc Groups from Other Sugar Chains

Novel complex-type oligosaccharides and glycopeptides with GalNAcβ1-4GlcNAcβ1–2 outer chains bind to a *W. floribunda* lectin (WFA)-Sepharose column ***(7).***

1. Equilibrate the WFA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Dissolve oligosaccharides or glycopeptides in 0.5 ml of lectin column buffer and apply to the column.
3. Elute 1.0-ml fractions with three column-volumes of lectin column buffer and then with three column-volumes of 100 mM *N*-acetylgalactosamine, at a flow rate of 2.5 ml/h at room temperature.
4. Collect other types of sugar chains, which pass through the column.
5. Collect complex-type sugar chains with GalNAcβ1-4GlcNAc outer chains, which elute after the addition of *N*-acetylgalactosamine.

3.4. Separation of High Mannose Type Sugar Chains from Complex Type and Hybrid Type Sugar Chains

3.4.1. Affinity Chromatography on Immobilized RCA

After the separation of high-molecular-weight poly-*N*-acetyllactosamine-type oligosaccharides, a mixture of the other three types of sugar chains can

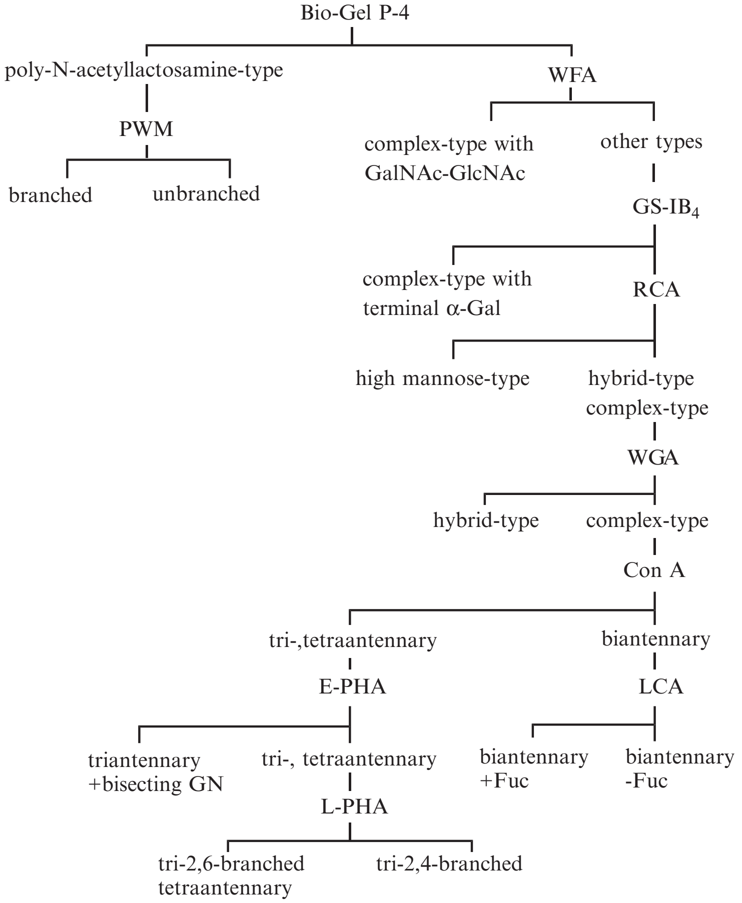
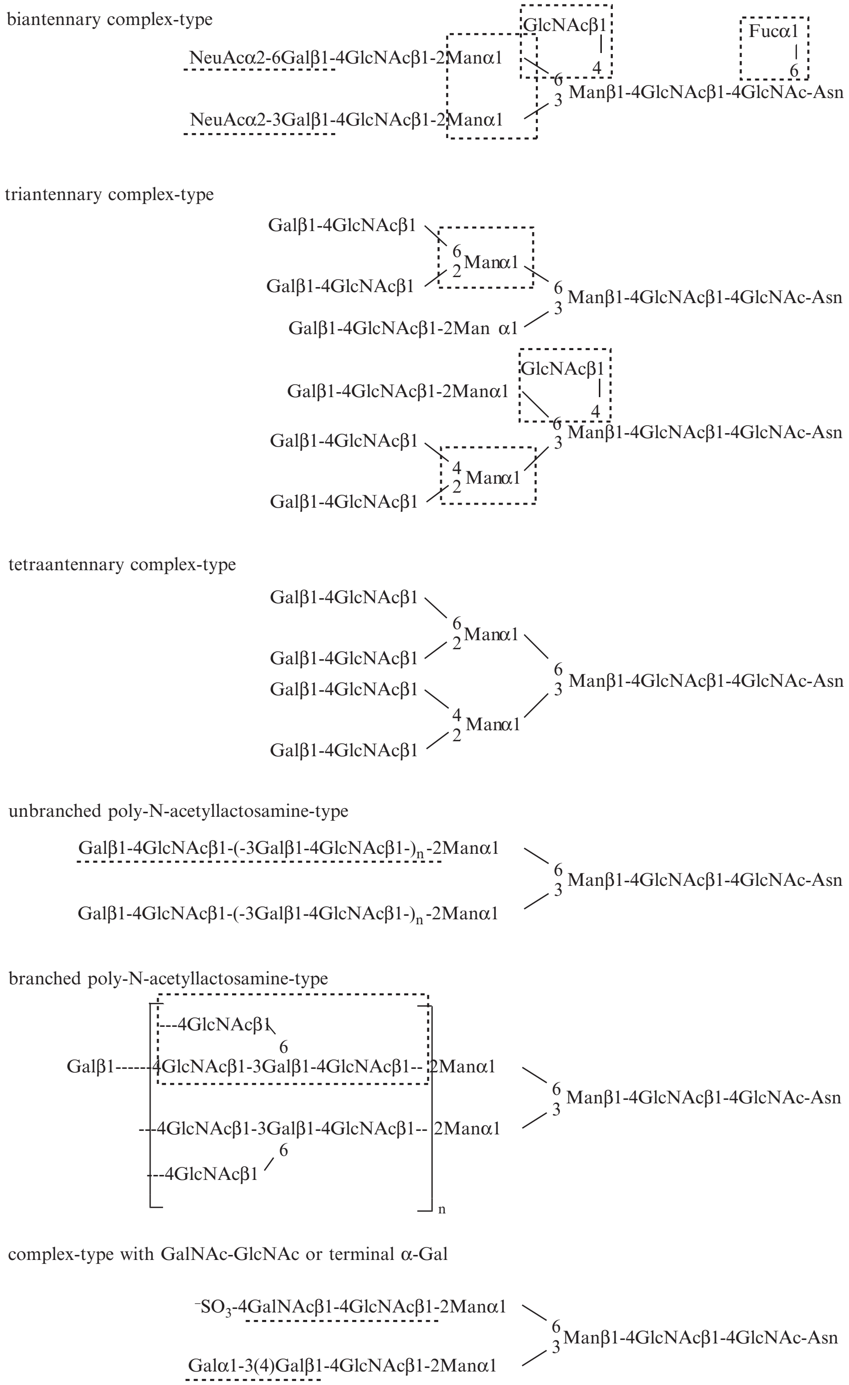

Bio-Gel P-4
poly-N-acetyllactosamine-type
WFA
PWM
complex-type with GalNAc-GlcNAc
other types
branched
unbranched
GS-IB$_4$
complex-type with terminal α-Gal
RCA
high mannose-type
hybrid-type complex-type
WGA
hybrid-type
complex-type
Con A
tri-,tetraantennary
biantennary
E-PHA
LCA
triantennary +bisecting GN
tri-, tetraantennary
biantennary +Fuc
biantennary -Fuc
L-PHA
tri-2,6-branched tetraantennary
tri-2,4-branched

Fig. 2. Fractionation scheme for asparagine-linked sugar chains by affinity chromatography with immobilized lectins

be separated on a column of *R. communis* lectin (RCA), which recognizes the Galβ1-4GlcNAc sequence *(17, 18).*

1. Equilibrate the RCA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Dissolve oligosaccharides or glycopeptides in 0.5 ml TBS and apply to the column.
3. Elute 1.0-ml fractions with three column-volumes of TBS, and then with three column-volumes of 50 mM lactose, at a flow rate of 2.5 ml/h at room temperature.
4. Bind both complex-type and hybrid-type sugar chains to the RCA-Sepharose column (*see* **Note 4**).
5. Collect high mannose-type oligosaccharides, which pass through the column.


biantennary complex-type

triantennary complex-type

tetraantennary complex-type

unbranched poly-N-acetyllactosamine-type

branched poly-N-acetyllactosamine-type

complex-type with GalNAc-GlcNAc or terminal α-Gal

GlcNAcβ1
4
Fucα1
6
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1
Galβ1-4GlcNAcβ1
6
Manα1
2
Galβ1-4GlcNAcβ1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
Galβ1-4GlcNAcβ1-2Man α1
GlcNAcβ1
Galβ1-4GlcNAcβ1-2Manα1
4
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
Galβ1-4GlcNAcβ1
4
Manα1
2
Galβ1-4GlcNAcβ1
Galβ1-4GlcNAcβ1
6
Manα1
2
Galβ1-4GlcNAcβ1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
Galβ1-4GlcNAcβ1
4
Manα1
2
Galβ1-4GlcNAcβ1
Galβ1-4GlcNAcβ1-(-3Galβ1-4GlcNAcβ1-)n-2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
Galβ1-4GlcNAcβ1-(-3Galβ1-4GlcNAcβ1-)n-2Manα1
---4GlcNAcβ1
6
Galβ1------4GlcNAcβ1-3Galβ1-4GlcNAcβ1-- 2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
---4GlcNAcβ1-3Galβ1-4GlcNAcβ1-- 2Manα1
6
---4GlcNAcβ1
n
⁻SO3-4GalNAcβ1-4GlcNAcβ1-2Manα1
6
3
Manβ1-4GlcNAcβ1-4GlcNAc-Asn
Galα1-3(4)Galβ1-4GlcNAcβ1-2Manα1

Fig. 3. Structures of several complex-type oligosaccharides. The boxed areas indicate the characteristic structures that are recognized by immobilized lectins.

6. Purify the oligosaccharides or glycopeptides from salts and haptenic sugar by gel filtration on a Sephadex G-25 column (1.2 cm id × 50 cm) equilibrated with distilled water.

3.4.2. Affinity Chromatography on Immobilized Snowdrop Lectin

High mannose-type glycopeptides, which carry Manα1-3Man units are specifically retarded on immobilized snowdrop (*G. nivalis*) lectin (GNA) ***(19).***

1. Equilibrate the GNA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Dissolve oligosaccharides or glycopeptides in 0.5 ml TBS and apply to the column.
3. Elute 0.5-ml fractions with five column-volumes of TBS to collect sugar chains lacking Manα1-3Man units or hybrid-type sugar chains, which are not retarded.
4. Elute with three column-volumes of 100 mM methyl-α-mannoside at a flow rate of 2.5 ml/h at room temperature to obtain the specifically retarded high mannose-type glycopeptides that carry Manα1-3Man units.

3.5. Separation of Hybrid Type Sugar Chains from Complex Type Sugar Chains

3.5.1. Affinity Chromatography on Immobilized WGA

Wheat germ lectin (WGA)-Sepharose has a high affinity for hybrid-type sugar chains. It has been demonstrated that the GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc-Asn structure is essential for tight binding of glycopeptides to a WGA-Sepharose column ***(20).***

1. Equilibrate the WGA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Dissolve glycopeptides in 0.5 ml TBS and apply to the column.
3. Elute 0.5-ml fractions with five column-volumes of TBS.
4. Collect the typical complex-type (and also high mannose-type) sugar chains eluted in the void volume of the column.
5. Collect hybrid-type glycopeptides with a bisecting *N*-acetylglucosamine residue, which are retarded on the WGA column.

3.6. Separation of Complex Type Biantennary Sugar Chains

3.6.1. Affinity Chromatography on Immobilized Concanavalin A (Con A)

Oligosaccharides and glycopeptides with tri- and tetraantennary complex-type sugar chains pass through Con A-Sepharose, whereas biantennary complex-type, hybrid-type, and high mannose-type sugar chains bind to Con A and can be differentially eluted from the column ***(21, 22).***

1. Equilibrate the Con A-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Pass the oligosaccharide mixture of complex-type chains from the WGA column through the Con A-Sepharose column.
3. Elute 1-ml fractions with three column-volumes of lectin column buffer.

4. Collect oligosaccharides with tri- and tetraantennary complex-type sugar chains, which pass through the column. Complex-type biantennary glycopeptides or oligosaccharides having bisecting GlcNAc also pass through the column.
5. Elute 1-ml fractions with three column-volumes of 10 mM methyl-α-glucoside and then with three column-volumes of 100 mM methyl-α-mannoside.
6. Collect complex-type biantennary sugar chains, which elute after the addition of methyl-α-glucoside.
7. Collect high mannose-type and hybrid-type oligosaccharides or glycopeptides eluted after the addition of methyl-α-mannoside.

3.6.2. Affinity Chromatography on Immobilized LCA, PSA, or VFA

The biantennary complex-type sugar chains bound to the Con A-Sepharose column and eluted with 10 mM methyl-α-glucoside include two types of oligosaccharides, which can be separated on a column of lentil (*L. culinaris*) lectin (LCA), pea (*P. sativum*) lectin (PSA), or fava (*V. fava*) lectin (VFA) ***(23–25)*** (*see* **Note 5**).

1. Equilibrate the LCA-, PSA-, or VFA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Pass the biantennary complex-type sugar chains from the Con A column through the LCA-, PSA-, or VFA-Sepharose column.
3. Elute 1.0-ml fractions with three column-volumes of lectin column buffer, followed by three column-volumes of 100 mM methyl-α-mannoside, at a flow rate of 2.5 ml/h at room temperature.
4. Collect biantennary complex-type sugar chains without fucose, which pass through the column.
5. Elute bound biantennary complex-type sugar chains having a fucose residue attached to the innermost *N*-acetylglucosamine.

3.6.3. Affinity Chromatography on Immobilized E-PHA

Complex-type biantennary sugar chains having outer galactose residues and bisecting *N*-acetylglucosamine are retarded by *P. vulgaris* erythroagglutinin (E-PHA)-Sepharose ***(18, 26).***

1. Equilibrate the E-PHA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Apply the pass-through fraction from the Con A column to the E-PHA-Sepharose column.
3. Elute 0.5-ml fractions with five column-volumes of lectin column buffer at a flow rate of 2.5 ml/h at room temperature.
4. Collect biantennary complex-type sugar chains having a bisecting *N*-acetylglucosamine residue, which were retarded on the E-PHA column (*see* **Note 6**). When the column is eluted at 4°C, biantennary complex-type oligosaccharides without a bisecting *N*-acetylglucosamine are also retarded by the E-PHA-Sepharose column.

3.7. Separation of Complex Type Triantennary and Tetraantennary Sugar Chains

3.7.1. Affinity Chromatography on Immobilized E-PHA

E-PHA-Sepharose interacts with high affinity with triantennary (having 2,4-branched mannose) oligosaccharides or glycopeptides containing both outer galactose residues and a bisecting *N*-acetylglucosamine residue **(26).**

1. Equilibrate the E-PHA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Apply the pass-through fraction from the Con A column to the E-PHA-Sepharose column.
3. Elute 0.5-ml fractions with five column-volumes of lectin column buffer at a flow rate of 2.5 ml/h at room temperature.
4. Collect retarded triantennary (having 2,4-branched mannose) oligosaccharides or glycopeptides containing both outer galactose and bisecting *N*-acetylglucosamine. Other tri- and tetraantennary oligosaccharides pass through the column (*see* **Note 7**).

3.7.2. Affinity Chromatography on Immobilized L-PHA

P. vulgaris leukoagglutinin (L-PHA), which is an isolectin of E-PHA, interacts with triantennary and tetraantennary complex-type glycopeptides having an α-linked mannose residue substituted at positions C-2 and C-6 with Galβ1-4GlcNAc (27).

1. Equilibrate the L-PHA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Apply the pass-through fraction from the Con A column to the L-PHA-Sepharose column.
3. Elute 0.5-ml fractions) with five column-volumes of lectin column buffer at a flow rate of 2.5 ml/h at room temperature.4. Collect retarded triantennary and tetraantennary complex-type glycopeptides having both 2,6-branched α-mannose and outer galactose (*see* **Note 8**). Other tri- and tetraantennary oligosaccharides pass through the column.

3.7.3. Affinity Chromatography on Immobilized DSA

D. stramonium lectin (DSA) has high affinity for tri- and tetraantennary complex-type oligosaccharides. Triantennary complex-type oligosaccharides containing 2,4-substituted α-mannose are retarded by the DSA-Sepharose column. Triantennary and tetraantennary complex-type oligosaccharides having an α-mannosyl residue substituted at the C-2 and C-6 positions bind to the column and are eluted by GlcNAc oligomers ***(28, 29).***

1. Equilibrate the DSA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the pass-through fraction from the Con A column to the DSA-Sepharose column.

3. Elute 0.5-ml fractions with three column-volumes of TBS at a flow rate of 2.5 ml/h at room temperature to obtain the retarded triantennary complex-type sugar chains having 2,4-branched α-mannose.
4. Elute with three column-volumes of 5 mg/ml *N*-acetylglucosamine oligomer at a flow rate of 2.5 ml/h at room temperature to obtain bound triantennary and tetraantennary complex-type oligosaccharides having an α-mannose residue substituted at the C-2 and C-6 positions.

3.8. Separation of Poly-*N*-Acetyllactosamine Type Sugar Chains

High-molecular-weight poly-*N*-acetyllactosamine-type oligosaccharides are classified into two groups: branched poly-*N*-acetyllactosaminoglycan containing a Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Gal unit and a linear poly-*N*-acetyllactosamine structure that lacks galactose substitutions at the C-3 and C-6 positions.

3.8.1. Affinity Chromatography on Immobilized PWM

Branched poly-*N*-acetyllactosamine-type oligosaccharides can be separated using a pokeweed mitogen (PWM)-Sepharose column ***(30).*** Since the sugar sequence Galββ1-4GlcNAcβ1-6Gal firmly binds to the PWM-Sepharose column, the branched poly-*N*-acetyllactosamine chains can be retained and the unbranched chains recovered without any retardation ***(31)*** (*see* **Note 9**).

1. Equilibrate the PWM-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the poly-*N*-acetyllactosamine-type sugar chains separated on Bio-Gel P-4 (*see* **Subheading 3.2**) to the PWM-Sepharose column.
3. Elute 1.0-ml fractions with three column-volumes of TBS, followed by three column-volumes of 0.1 M NaOH, at a flow rate of 2.5 ml/h at room temperature.
4. Collect the unbranched poly-*N*-acetyllactosamine-type sugar chains, which pass through the column.
5. Collect the bound branched poly-*N*-acetyllactosamine-type sugar chains.

3.8.2. Affinity Chromatography on Immobilized DSA

Immobilized DSA lectin interacts with high affinity with sugar chains having a linear, unbranched poly-*N*-acetyllactosamine sequence. For binding to DSA-Sepharose, more than two intact *N*-acetyllactosamine repeating units may be essential ***(29).***

1. Equilibrate the DSA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the poly-*N*-acetyllactosamine-type sugar chains separated on Bio-Gel P-4 (*see* **Subheading 3.2**) to the DSA-Sepharose column.
3. Elute 1.0-ml fractions with three column-volumes of TBS, followed by three column-volumes of 5 mg/ml GlcNAc oligomer, at a flow rate of 2.5 ml/h at room temperature.
4. Collect branched poly-*N*-acetyllactosamine-type sugar chains, which pass through the column and are separated from unbranched poly-*N*-acetyllactosamine-type sugar chains, which bind to the column.

3.9. Separation of Terminally Sialylated or β-galactosylated sugar chains

The basic Galβ1-4GlcNAc sequence present in complex-type sugar chains usually contains sialic acids in α2,6 or α2,3 linkages in the outer galactose residues.

3.9.1. Affinity chromatography on immobilized MAL

M. amurensis leukoagglutinin (MAL) ***(32, 33)*** interacts with high affinity with complex-type tri- and tetraantennary glycopeptides containing an outer sialic acid residue linked α2,3 to the penultimate galactose. Glycopeptides containing sialic acid linked only α2,6 to galactose do not interact detectably with the immobilized MAL (*see* **Note 10**).

1. Equilibrate the MAL-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Apply the acidic oligosaccharides or glycopeptides separated on Mono Q or DEAE-Sepharose (*see* **Subheading 3.1.1**, step 1) to the MAL-Sepharose column.
3. Elute 0.5-ml fractions with five column-volumes of lectin column buffer at a flow rate of 2.5 ml/h at room temperature.
4. Collect glycopeptides or oligosaccharides containing α2,6-linked sialic acid(s), which pass through the column.
5. Collect retarded glycopeptides or oligosaccharides containing α2,3-linked sialic acid(s).

3.9.2. Affinity Chromatography on Immobilized Allo A

A. dichotoma lectin (allo A) (34, 35) recognizes the other isomer of sialyllactosamine. Mono-, di-, and triantennary complex-type oligosaccharides containing terminal sialic acid(s) in α2,6 linkages bind to allo A-Sepharose, whereas complex-type sugar chains having isomeric α2,3-linked sialic acid(s) do not bind to immobilized allo A.

1. Equilibrate the allo A-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the acidic oligosaccharides or glycopeptides separated on Mono Q or DEAE-Sepharose (*see* **Subheading 3.1.1,** step 1) to the allo A-Sepharose column.
3. Elute 0.5-ml fractions with three column volumes of TBS and then with three column-volumes of 50 mM lactose at a flow rate of 2.5 ml/h at room temperature.
4. Collect glycopeptides or oligosaccharides containing α2,3-linked sialic acid(s), which pass through the column.
5. Elute bound glycopeptides or oligosaccharides having α2,6-linked sialic acid(s) (*see* **Note 11**).

3.9.3. Affinity Chromatography on Immobilized SNA

Elderberry (*S. nigra L.*) bark lectin (SNA) ***(36, 37)*** has the same sugar-binding specificity as allo A. All types of oligosaccharides with at least one NeuAcα2-6Gal unit bind firmly to SNA-Sepharose.

1. Equilibrate the SNA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the acidic oligosaccharides or glycopeptides separated on Mono Q or DEAE-Sepharose (*see* **Subheading 3.1.1,** step 1) to the SNA-Sepharose column.
3. Elute 0.5-ml fractions with three column-volumes of TBS, followed by three column-volumes of 50 mM lactose, at a flow rate of 2.5 ml/h at room temperature.
4. Collect glycopeptides or oligosaccharides containing α2,3-linked sialic acid(s), which pass through the column.
5. Bound glycopeptides or oligosaccharides having α2,6-linked sialic acid(s) elute in the 50 m*M* lactose.

3.9.4. Affinity Chromatography on Immobilized GS-IB$_4$

Griffonia (Bandeiraea) simplicifolia isolectin B$_4$ (GS-IB$_4$) binds specifically and with high affinity to complex-type sugar chains containing terminal Galα1-3Gal or Galα1-4Gal sequences ***(8, 38).*** Although *G. simplicifolia* isolectin A$_4$ (GS-IA$_4$) and GS-IB$_4$ bind α-galactosyl groups with equal affinity, B$_4$ has a K$_a$ for α-*N*-acetylgalactosaminyl groups that is three orders of magnitude smaller than that of A$_4$.

1. Equilibrate the GS-IB$_4$-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the neutral oligosaccharides or glycopeptides onto the GS-IB$_4$-Sepharose column.
3. Elute 0.5-ml fractions with three column-volumes of TBS, followed by three column volumes of 100 mM methyl α-galactoside, at a flow rate of 2.5 ml/h at room temperature.
4. Collect glycopeptides or oligosaccharides without the Galα1-3(4)Gal sequence, which pass through the column.
5. Retarded or bound glycopeptides or oligosaccharides having α-galactosyl residue(s) elute in the 100 m*M* methyl α-galactoside eluant.

4. Notes

1. During the coupling reactions, sugar-binding sites of lectins must be protected by the addition of the specific haptenic sugars.
2. Immobilized lectin is stored at 4°C. In most cases, immobilized lectin is stable for several years.
3. Some lectins, especially leguminous lectins, need Ca^{2+} and Mn^{2+} ions for sugar binding. Therefore, the buffers used for affinity chromatography on the lectin column should contain 1 mM $CaCl_2$ and 1 mM $MnCl_2$.
4. Complex-type and hybrid-type oligosaccharides are retarded on, rather than tightly bound to, a RCA-Sepharose column when their sugar sequences are masked by sialic acids or α-galactosyl residues.
5. Intact *N*-acetylglucosamine and asparagine residues at the reducing end are required for tight binding of complex-type oligosaccharides to LCA-, PSA-, or VFA-Sepharose columns.

6. High-affinity interaction with E-PHA-Sepharose is prevented if both outer galactose residues on a bisected sugar chain are substituted at position C-6 by sialic acid.
7. Biantennary and triantennary complex-type sugar chains having bisecting GlcNAc can be separated on a Bio-Gel P-4 column.
8. L-PHA-Sepharose does not retard the elution of sugar chains lacking outer galactose residues.
9. WGA can be used instead of PWM.
10. *M. amurensis* hemagglutinin (MAH), which is an isolectin of MAL, binds strongly to sialylated Ser/Thr-linked Galβ1-3GalNAc, but not to sialylated Asn-linked sugar chains ***(38).***
11. Mono-, di-, tri-, and tetraantennary complex-type oligosaccharides without sialic acid(s) are retarded by the allo A lectin column.
12. More detailed reviews on the separation of oligosaccharides and glycopeptides by means of affinity chromatography on immobilized lectin columns have been published ***(39, 40).***

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In-Gel Enzymatic Release of *N*-Glycans

David J. Harvey

1. Introduction

Analysis of *N*-linked glycans, those attached to Asn in an Asn-Xxx-Ser(Thr) motif, where Xxx is any amino acid except proline, requires determination of which consensus sequence is occupied, the extent of occupancy and the structures of the glycans that are attached to each site. Structural analysis of the glycans is most commonly performed following their removal, either chemically ***(1,2)*** or enzymatically, and this chapter describes an enzymatic method for glycan removal using protein *N*-glycosidase F (PNGase F) ***(3)*** from within the SDS-PAGE gels commonly used to separate and purify the glycoproteins. The method is a modification of that described by Küster *et al*. ***(4)*** in 1997. It can be used for mammalian glycans but not for those that are substituted at the 3-position of the reducing terminal GlcNAc residue, commonly found in plants and insects. These glycans are resistant to PNGase F but can be released with PNGase A after trypsinolysis of the protein. Released glycans can then be examined directly by mass spectrometry, usually with MALDI or electrospray ionization, or they can be labeled at their reducing terminus for analysis by chromatographic or electrophoretic techniques.

2. Materials

2.1. Apparatus

2.1.1. Electrophoresis and Glycan Release

1. BioRad Mini-Protean II apparatus with 80 × 80 × 0.75 mm plates, or similar.
2. 500 µL Eppendorf tubes.
3. Temperature-controlled heating block.
4. BioRad Spin column or equivalent.
5. Plastic hypodermic-type syringe (1 mL).
6. Millipore 0.5 µm FH-type filters.

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2.1.2. Optional Glycan Purification

1. GelLoader® pipette tips.
2. Supply of nitrogen.

2.2. Chemicals

2.2.1. Electrophoresis and Glycan Release

1. Protein molecular weight markers.
2. Reference glycoprotein (e.g., ovalbumin. ribonuclease B)
3. Aluminium foil.
4. Protein *N*-glycosidase F e.g. 1000 U/mL recombinant glycerol-free lyophilisate (Roche Life Sciences) (*see* **Note 1**).
5. Dowex AG50Wx12 Resin (H^+ form; 100 to 200 mesh; Bio-Rad).
6. pH Test paper (to check for neutrality).

2.2.2. Optional Glycan Purification

1. Dowex AG3x4 resin
2. C-18 resin (*see* **Note 2**).
3. Dimethylsulfoxide (DMSO).
4. Methyl iodide.
5. Sodium hydroxide.

2.2.3. Solutions

1. 17.5% Resolving gel for proteins in the range 10–70 kDa (*see* **Note 3**): 87.5 mL of 30% acrylamide, 5.5 mL of 2% bis-acrylamide 50.5 mL of 1 M Tris buffer (pH 8.8, acetic acid), 0.75 mL of 20% SDS and add water to give a total volume of 150 mL.
2. 12.5% Resolving gel for proteins in the range 70–200 kDa: 62.5 mL of 30% acrylamide, 15.5 mL of 2% bis-acrylamide 56.0 mL of 1 *M* Tris buffer (pH 8.8, acetic acid), 0.75 mL of 20% SDS and add water to give a total volume of 150 mL.
3. Resolving gel polymerization mixture: 18 µL TEMED and 33 µL of 10% APS.
4. Stacking gel: 20.0 mL of 30% acrylamide, 7.8 mL of 2% bis-acrylamide 15.0 mL of 1 *M* Tris buffer (pH 8.8, acetic acid), 0.6 mL of 20% SDS and add water to give a total volume of 120 mL.
5. Stacking gel polymerization mixture: 12 µL TEMED and 25 µL of 10% APS.
6. Loading buffer: 5 mL 2 *M* ammonium formate, pH 4.4; 50 mL Milli-Q-purified water, Adjust pH to 8.6, Add 0.4 g SDS and water to a final volume of 100 mL.
7. Electrophoresis running buffer: 25 m*M* Tris, 190 m*M* glycine, 0.5% SDS.
8. Destain solution: 5% methanol/7% acetic acid.
9. Incubation buffer: 20 m*M* $NaHCO_3$, pH 7.0; 336 mg $NaHCO_3$ in 200 mL Milli-Q water, adjust to pH 7.0 with acetic acid.
10. 45 mM DTT solution: 6.9 mg of DDT in 1mL of $NaHCO_3$ buffer.
11. Iodoacetamide solution: 18.5 mg of iodoacetamide in 1 mL of $NaHCO_3$ buffer (*see* **Note 4**).

12. PNGase-F solution: 50 Units of PNGase F powder in 0.25 mL Milli-Q water (200 Units/mL and dialyse into 20 m*M* $NaHCO_3$ buffer (*see* **Note 5**). Add 50 µL distilled water to give a final concentration of 100 Units/mL.

3. Methods

3.1. Gel Electrophoresis

3.1.1. Preparation of Gel

1. Mix 5 mL of the resolving gel mixture with the polymerization mixture and pour to 1 cm below the top of the electrophoresis cell.
2. Overlay with about 2 mm of *iso*-butanol/water and leave the gel to set for about 30 min.
3. Wash off *iso*-butanol/water and remove excess water.
4. Mix 5 mL of the stacking gel mixture with the polymerization mixture and pour onto the resolving gel to fill the cell.
5. Insert the comb into the stacking gel, avoiding air bubbles, and allow the mixture to set for 30 mins.

3.1.2. Preparation of Sample

1. Mix the sample, which should contain about 50–100 pmol of glycoprotein with twice its volume of loading buffer and bromophenol blue.
2. Boil for 5 mins in a hot block (in an uncapped tube), cool and spin in a microcentrifuge.

3.1.3. Running of Gel

1. Clip the cell containing the gel into its frame and place into the electrophoresis tank.
2. Add running buffer to the tank and remove any air bubbles.
3. Remove the comb from the stacking gel and wash the wells with Milli-Q water.
4. Fill the spaces with running buffer.
5. Add bromophenol blue dye to the outer lanes.
6. Add 3 µL of molecular weight markers to the next lanes.
7. Add a reference glycoprotein (e.g. ovalbumin 10 µL, 100 pmol, 4.5 mg) + dye to one lane.
8. Load the samples (10 µL) into the remaining lanes (*see* **Note 6**).
9. Fill empty lanes with running buffer.
10. Connect to the power supply (200 V).
11. Switch on and set the current to about 50 mA.
12. Allow to run until the dye runs out at the bottom of the gel (*see* **Note 7**).

3.1.4. Gel Staining

1. Empty the tank, remove the gel in its cell and wash with tap water.
2. Remove the spacers, lever apart the glass plates and remove the stacking gel.
3. Mark the top of the gel by cutting off a corner.
4. Wash the gel from the remaining glass plate with a stream of water into a plastic box.

5. Cover the gel with Coomassie blue solution, cover and shake for 10–15 mins
6. Pour away the dye solution.

3.1.5. Gel Destaining

1. Wash the gel with tap water.
2. Cover the gel with the destain solution (*see* **Note 8**) and shake for 10 min
3. Change the destain solution and shake for a further 10 mins.
4. Repeat step 3.
5. Cut out the bands from the gel that contains the target glycoproteins. (*see* **Note 9**) and place into the Eppendorf tube with a small amount of destain solution.
6. Place the tube in a shaker overnight at room temperature.
7. Wash gel pieces with 300 µL of incubation buffer, vortex, centrifuge briefly, and soak for 30 min at room temperature. Discard the wash.
8. Repeat step 7.
9. Wash the gel pieces for 60 min in 300 µL of 1:1 acetonitrile: 20 mM $NaHCO_3$ to remove residual SDS.
10. Dry gel pieces in a vacuum centrifuge.

3.2. Glycan Removal

3.2.1. Reduction of Glycoprotein

1. Add 300 µL of incubation buffer (to cover gel bits).
2. Add 20 µL of the DTT solution.
3. Cap, spin and heat in a shaker at 60°C for 30 mins.

3.2.2. Alkylation of Glycoprotein

1. Add 20 µL of the iodoacetamide solution to the gel-containing tube.
2. Wrap the tube in aluminium foil and shake for 30 min at room temperature.
3. Remove the buffer and iodoacetamide.
4. Add about 0.5 mL of $NaHCO_3$ buffer and 0.5 mL of acetonitrile and shake at room temperature for 1 hour.
5. Remove the buffer and acetonitrile.
6. For each sample, cut the gel piece into small bits and place the gel bits into a 500 µL Eppendorf tube that has holes pierced in the lid.
7. Place the tubes in a SpeedVac (continuous rotor) until they are dry (crisp).

3.2.3. Removal of Sugars with PNGase-F

1. Add 30 µL of the PNGase solution to each sample (the solution should just cover the gel bits).
2. Leave at room temperature until all liquid has been taken up by the gel (20–30 mins.)
3. Add 150 µL of $NaHCO_3$ buffer.
4. Incubate at 37°C overnight.

3.2.4 Extraction of N*-Glycans from the Gel*

1. Prepare AG50 (30 µL/sample) by adding the AG50 resin (*see* **Note 10**) to a BioRad BioSpin column, running approx. 10 col. volumes of HCl (1:1, v:v dilution of concentrated HCl) through the AG50 and then washing with Milli-Q water until the washings are neutral (about pH 5 as tested by pH paper).
2. Add 100 µL of distilled water to each sample and sonicate for 30 mins.
3. Remove the solution and add to a small screw-cap tube containing the AG50 resin.
4. Repeat steps 2 and 3 twice, adding the solution to the tube containing the resin.
5. Add 200–300 µL of acetonitrile to each sample and sonicate for 30 mins. (*see* **Note 11**).
6. Remove the acetonitrile and add to the tube containing the resin.
7. Shake the resin-containing tube and spin in a microcentrifuge.
8. Filter through a 0.5 µm FH-type Millipore filter attached to a 1 mL plastic syringe.
9. Wash the resin, filter the washings and add to the first filtrate
10. Dry the sample in a SpeedVac.
11. Add 20 µL 1% formic acid and allow to stand at room temperature for 40 mins (*see* **Note 12**).
12. Dry in a SpeedVac.

3.2.5. Removal of Contaminants from the Glycans

The samples will still be contaminated with, possibly, peptides, salts, SDS etc. that must be removed before analysis by mass spectrometry or HPLC. Several methods are available. We prefer the Nafion membrane procedure ***(5)*** that is described in the chapter on the analysis of *N*-glycans by mass spectrometry. However, a second stage of preliminary cleaning may be required. For neutral glycans (*see* **Note 13**), this can be achieved as follows:

1. Fill a GelLoader tip with ethanol.
2. Add a few µL of G3 × 4 resin, followed by washed AG50 resin and C-18 resin to the GelLoader tip to form three layers.
3. With nitrogen from a cylinder, force ethanol through column until just to the top of the resin (*see* **Note 14**).
4. Add 100 µL of Milli-Q water to the top of the resin "sandwich" and force the water through the resins with the nitrogen.
5. Repeat the washing procedure twice.
6. Add the aqueous sample solution to the resin column.
7. Force the solution through the resins with nitrogen and collect into a small Eppendorf (PCR) tube.
8. Add 50 µL Milli-Q water to the resins, blow through and add to the glycan solution.
9. Dry the glycans in a Speedvac.

3.2.6. Sialylated Glycans

Sialylated glycans will be removed by the AG3 resin in the above procedure. However they can be neutralized (and stabilized for analysis by MALDI mass spectrometry) by formation of methyl esters prior to clean-up by the three-bed resin procedure.

1. Place about 100 µL AG5OW × 12 resin in a short column.
2. Wash with 1 mL of 1 M sodium hydroxide solution followed by water until the washings are approximately neutral.
3. Dissolve the glycan sample (100–500 pmole) in about 5–20 µL water and apply to the column.
4. Wash through with 5–100 µL of water and collect the washings in a small Eppendorf tube.
5. Dry in a rotary evaporator.
6. Dissolved the glycans in about 1 µL of dry DMSO and add 1 µL of methyl iodide (*see* **Note 15**).
7. Mix well and allow to stand for 2 h at room temperature.
8. Add about 10 mL of DMSO and evaporate the methyl iodide with a stream of nitrogen.
9. Evaporate to dryness (*see* **Note 16**).

4. Notes

1. Preparations containing glycerol should be avoided.
2. C18 resin can be obtained from a C18 SepPak cartridge
3. Higher bis-acrylamide concentrations lead to increased cross-linking and reduced glycan yield.
4. Iodoacetamide is light sensitive and solutions should be kept in the dark.
5. It is best not to use nonammonium-containing buffers at this stage. PNGase F releases the glycans as the glycosylamine that need to be converted into the free sugars before further analysis. Ammonium-containing buffers promote glycosylamine retention ***(6)***.
6. It is a good idea to load the samples asymmetrically so that it is possible to determine the left and right sides of gel.
7. The current normally drops to about 10 mA.
8. A low concentration of acetic acid is used to minimizes loss of sialic acid from the glycans.
9. The gel pieces should be small but wide enough to retain minor glycoforms.
10. The resin, which should be stored in water, can be handled with a Gilson pipette fitted with a blue or yellow tip that has been cut to widen the end.
11. The acetonitrile causes the gel to shrink, expelling the remaining water.
12. Because the glycans are released as glycosylamines, a considerable amount of the sample may be in this form at this stage. The glycosylamines can

be detected by mass spectrometry by their mass being one unit less than that of the native glycan. If labelling of the reducing terminus is attempted at this stage, the glycosylamines will not react, resulting in a poor yield of labeled material. Furthermore, the rate and extent of glycosylamine hydrolysis to the glycan depends on whether or not there is a fucose residue at the reducing terminus. Consequently, samples must be fully converted into their native glycan (OH) form before analysis is attempted. Conversion can be achieved by leaving the glycans to stand in water or, better, in dilute formic acid. However, care must be taken to avoid hydrolysis of sensitive bonds such as those linking the sialic acids.

13. The AG3 resin is included in this clean-up procedure mainly to remove any residual SDS. However, it will also remove acidic (sialylated, sulfated glycans). To prevent sialic acid removal, the sialic acids can be converted into methyl esters as described by Powell and Harvey *(7)* and in Subheading 3.2.6 before the 3-resin method is applied. However, sulfates cannot be methylated by this method.
14. In this and subsequent steps, release the nitrogen pressure when the top surface of the liquid reaches the top of the resins in order to avoid introducing air bubbles to the resin.
15. Methyl iodide is toxic; consequently, this procedure should be performed in a fume hood.
16. DMSO is not very volatile, consequently, evaporation is slow.

Acknowledgments

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Analysis of *N*-Linked Glycans by Mass Spectrometry

David J. Harvey

1. Introduction

Analysis of *N*-linked glycans, those attached to Asn in an Asn-Xxx-Ser(Thr) motif, where Xxx is any amino acid except proline, involves determination of which consensus sequence is in fact occupied by glycans, the extent of occupancy and the structures of the glycans that are attached. This chapter deals only with a small set of these procedures, namely the structural analysis of the carbohydrates using mass spectrometry as the main analytical technique. It is assumed that the glycans have been released from the glycoprotein or derived glycopeptide by techniques such as hydrazinolysis ***(1,2)*** or enzymolysis with an endoglycosidase such as protein-*N*-glycosidase F (PNGase F) ***(3,4)*** and that the released glycans are available in aqueous solution. Traditionally, such mixtures of glycans would be labeled with a chromophore or fluorophore such as 2-aminobenzamide ***(5)*** and profiled by gel-filtration or high-performance liquid chromatography (HPLC) with structures being deduced by subsequent profiling of the products of sequential exoglycosidase digestion with a library of exoglycosidases. Knowledge of the enzyme specificity and the number of constituent monosaccharide residues removed at each stage of the procedure allows the structure of the glycan(s) to be deduced. The method, although valuable to the mass spectroscopist for providing information on the nature of the constituent monosaccharides, is limited by the range, availability and purity of the available exoglycosidases which does not include enzymes appropriate for all structural features.

Mass spectrometry, although capable of rapidly supplying much of the information that is difficult to obtain by the exoglycosidase technique, still relies on

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it for confirmation of the identity of the constituent monosaccharides. However, because of the very conserved nature of the *N*-glycan biosynthetic pathway and because much work on the analysis of *N*-glycans is performed with species with well characterized enzymology, mass measurements are often sufficient to provide the structural information that is required. This information relates to questions such as what type of glycan (high-mannose, hybrid, complex) is present and the branching pattern and composition of the antennae. However, for rigorous work, it must be remembered that a mass measurement will only classify monosaccharide constituents into groups such as hexose, HexNAc, and so on.

The ideal mass spectrometric approach is one in which the initial spectrum produces the glycan profile, preferably quantitatively, with no fragmentation and subsequent spectra give extensive fragmentation to provide the detailed structural information required on each component. Glycan profiles are conveniently obtained by matrix-assisted laser desorption/ionization (MALDI) mass

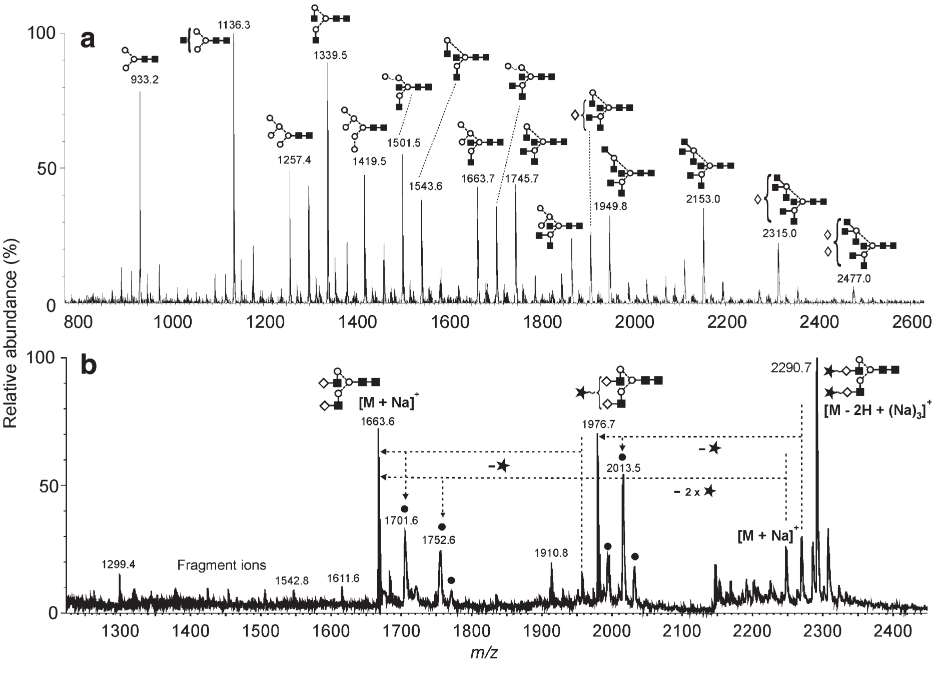

Fig. 1. **(a)** Positive ion MALDI-TOF mass spectrum of *N*-glycans released from a mixture of glycoproteins (mainly ovalbumin) from chicken egg white. **(b)** Positive ion reflectron MALDI-TOF spectrum of a disialylated biantennary glycan. Peaks annotated with a black spot are metastable (PSD) ions. Key to symbols used for the constituent monosaccharides in this scheme and subsequent Figs. ◊ = *galactose,* □ = *GlcNAc*, □ = *mannose,* □ = *fucose,* □ = *N-acetylneuraminic* (sialic) acid. Solid lines connecting the symbols are β-bonds and broken lines are α-bonds.

spectrometry ***(6–9)***, a technique that gives a quantitative response and negligible fragmentation for neutral *N*-glycans of varying structure ***(10,11)*** **(Fig. 1a)**. Sialylated glycans, however, tend to fragment by loss of sialic acid **(Fig. 1b)** but can be neutralized and stabilized by methyl ester formation ***(12)***. The somewhat milder technique of electrospray ionization (ESI) is being increasingly used but tends to produce ions in different charge states that limit its usefulness as a profiling technique. It is, however, an ideal method for introducing glycans into a mass spectrometer for fragmentation analysis. Older, less sensitive techniques such as fast-atom bombardment (FAB) mass spectrometry have also been widely used for glycan analysis ***(13,14)*** but produce extensive fragmentation, require derivatization of the glycans and are now mainly of historical interest.

The mass of the glycan provides a direct indication of the constituent monosaccharide composition in terms of sugar type (hexose, deoxy-hexose, and so on) because most *N*-glycans consist of only a limited number of mass-different monosaccharides. **Table 1** lists the residue masses for these monosaccharides. Suitable software is available on the internet at https://tmat.proteomesystems.

Table 1
Residue Masses of Common Monosaccharides Found in *N*-Glycans

Monosaccharide	Residue formula	Residue mass[a]
Pentose	$C_5H_8O_4$	132.042 132.116
Deoxy-hexose	$C_6H_{10}O_4$	146.078 146.143
Hexose	$C_6H_{10}O_5$	162.053 162.142
Hexosamine	$C_6H_{11}NO_4$	161.069 161.157
HexNAc	$C_8H_{13}NO_5$	203.079 203.179
Hexuronic-Acid	$C_6H_8O_6$	176.032 176.126
N-Acetyl-neuraminic acid	$C_{11}H_{17}NO_8$	291.095 291.258
N-glycoyl-neuraminic acid	$C_{11}H_{17}NO_9$	307.090 307.257

a) Top Fig. = monoisotopic mass (based on C = 12.000000, H = 1.007825, N = 14.003074, O = 15.994915), Lower Fig. = average mass (based on C = 12.011, H = 1.00794, N = 14.0067, O = 15.9994.

The masses of the intact glycans can be obtained by addition of the residue masses given above, plus the mass of the terminal group (H_2O for an unmodified glycan) 18.011 (monoisotopic) and 18.152 (average) and the mass of any reducing-terminal or other derivative.

com/glyco/glycosuite//glycodb (requires password) for deriving compositions from experimental masses. Once the monosaccharide composition is established, structural features can be assigned by fragmentation. Fragmentation from MALDI-generated ions produced in time-of-flight (TOF) instruments can be obtained by analysis of spontaneously produced post-source decay (PSD) ions ***(15)*** but resolution is generally poor and many commercial instruments require the spectra to be recorded in segments with consequent reduction in quantitative information. Greatly improved performance can be obtained by generating the fragments by collision in a tandem instrument such as a quadrupole/time-of flight (Q-Tof) mass spectrometer ***(16–20)***. Multiple successive fragmentations can be produced with a storage instrument such as an ion trap ***(21–23)*** and can be used to extract detailed structural information from small fragments of the glycan.

The information that can be obtained from the spectrum depends to a large extent on the type of ion that is initially formed. MALDI Produces mainly $[M+Na]^+$ ions that fragment predominantly by cleavage between the sugar rings (glycosidic cleavage) to provide sequence information but little linkage data. Linkage information is provided by cross-ring cleavage fragments that are usually present in only low relative abundance in low-energy spectra of the type produced by trap and Q-Tof-type instruments. Cross-ring fragments of higher abundance are found in the spectra recorded with instruments providing higher-energy collisions ***(24–26)***. Unfortunately, fragmentation spectra of $[M+Na]^+$ ions contain ambiguous information because fragment ions of the same mass and composition often arise from different regions of the molecule. The situation is even more difficult with the $[M+H]^+$ ions frequently encountered in ESI spectra **(Fig. 2a);** fragmentation of these ions rarely yield cross-ring cleavages and are prone to internal rearrangements ***(27–30)*** that again yield ambiguous information.

Negative ion spectra, on the other hand, are much more informative because cross-ring cleavage reactions are frequently dominant ***(31–37)*** **(Fig. 2)** and the

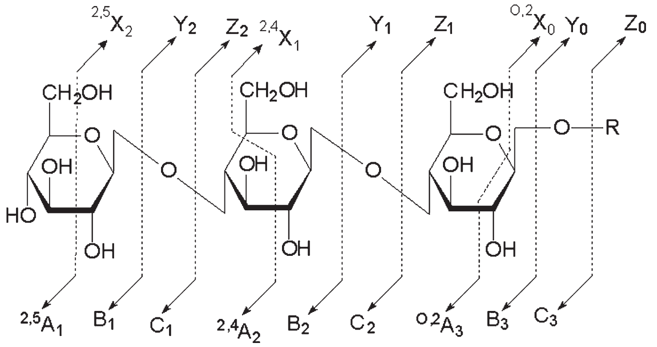

Fig. 2. Nomenclature for describing fragment ions from carbohydrates according to Domon and Costello ***(39).***

redundancy seen in the positive ion spectra is largely absent. Spectra of isomeric compounds are usually different in that they produce ions of different masses unlike positive ion spectra where the presence of isomers is often only reflected in differences in ion abundance. Consequently, the spectra contain ions that are diagnostic of specific structural features that cannot be obtained easily from the positive ion spectra or from the more classical analytical methods and form the basis of this chapter. The protocol described below is one that is used in our laboratory to provide most of the structural information necessary to analyse complex mixtures of *N*-glycans that have been released with PNGase from glycoproteins separated in SDS-PAGE gels ***(4)***.

2. Materials

2.1. Purification of Glycans

1. Nafion 117 membrane (Aldrich Chemical Co. Ltd., Poole, UK).
2. 35% Nitric acid.
3. Milli-Q water.

2.2. MALDI Mass Spectrometry

1. Matrix solution (saturated solution of dihydroxybenzoic acid (DHB) in acetonitrile (*see* **Note 1**).
2. Ethanol.

2.3. Electrospray Mass Spectrometry (Negative Ion)

1. Ammonium phosphate.
2. Nanospray capillaries (Proxeon).
3. Methanol:Milli-Q water (1:1, v:v).
4. Gel-Loader pipette tips (Eppenforf 20 μL).

3. Methods

3.1. Purification of Glycans

Most glycan solutions contain residual salt, peptides etc. whose presence will degrade the quality of the mass spectrum or completely suppress the signals from the glycans. Normally neutral glycans give much weaker signals than peptides on account of their different ionization properties (formation of $[M+Na]^+$ rather than $[M+nH]^{n+(-)}$ ions. If such contamination is expected, proceed with a clean-upstage as follows. If not, this stage can be omitted. The method is essentially as described by Börnsen *et al.* ***(38)***.

1. Condition small squares (about 3 cm square) of the Nafion 117 membrane by heating in 35% nitric acid for 4 hours at 80°C.
2. Wash each square thoroughly with Milli-Q water (*see* **Note 2**).

3. Dry a square of membrane with filter paper and float it on the surface of Milli-Q water in a small shallow container.
4. Deposit 1 µL (or similar) drops of the aqueous glycan solutions (*see* **Note 3**) onto the membrane surface and allow it to sit for about 20 mins. (*see* **Note 4**).
5. Remove the glycan solution with a pipette and either deposit directly onto the MALDI target or dilute for ESI analysis as described below.

3.2. MALDI Mass Spectrometry

1. Deposit 0.5–1 µL glycan solution onto the MALDI target.
2. Using a separate pipette tip, add 0.3 µL matrix solution and allow the mixture to dry at room temperature. Inspect the dried sample deposit. (*see* **Notes 5 and 6**).
3. If good crystals are present, redissolve the spot in the minimum amount of ethanol and allow it to recrystalize (*see* **Note 7**).
4. Introduce the target into the mass spectrometer and acquire both positive and negative ion spectra in reflectron mode (*see* **Note 8**). Keep the laser power as low as practical in order to avoid peak broadening and consequent mass errors (*see* **Note 9**) and frequently move the laser spot to different areas of the target (*see* **Note 10**) until a satisfactory signal:noise ratio is obtained (*see* **Note 11**).
5. If sialic acids are detected, they can be stabilized by forming methyl esters using the protocol described in the chapter on in-gel release of glycans and reference ***(12)*** (*see* **Note 12**).
6. If desired, recover the remaining sample from the MALDI target after MS analysis by dissolving the spot in several repetitive additions of 2 µL of 1:1 (v:v) methanol:water and removing with a pipette. The matrix can then removed from the resultant solution by drop dialysis or use of a C18 ZipTip.

3.3. Electrospray Mass Spectrometry (Negative Ion)

1. Dilute the cleaned glycan solution with about 5 µL of 1:1 (v:v) methanol:water containing a trace of ammonium phosphate (*see* **Note 13**).
2. Introduce the glycan solution into the nanospray capillary with a 20 µL pipette fitted with a GelLoader pipette tip (*see* **Note 14**).
3. Introduce the capillary into the mass spectrometer with the voltage set to zero, introduce a small pressure on the capillary and start the flow by touching the tip of the capillary on the cone. If the spray does not start immediately, scrape the tip on the cone to try and break off the end.
4. Once the flow has started, turn up the capillary voltage to about 1.1 kV (Waters-Micromass Q-Tof Ultima Global instrument. Other instruments may vary).
5. Record the MS spectrum and then the MS/MS spectra of each relevant ion (*see* **Notes 15** and **16**).

3.4. Spectral Interpretation

3.4.1. Neutral Glycans

The *N*-glycans fragment by glycosidic (between the monosaccharide rings) and cross-ring cleavages. All glycosidic cleavages are accompanied by hydrogen migration to give the product even-electron ion and a neutral molecule. The nomenclature used to describe the fragmentation was proposed by Domon and Costello in 1988 ***(39)*** and has been universally accepted. It is outlined in **Fig. 2**. Ions retaining the charge on the reducing terminus are designated *X*, *Y*, and *Z* where *X* is a cross ring cleavage and *Y* and *Z* are glycosidic cleavages depending on which side of the linking oxygen the bond is broken. Subscript numbers designate the position along the chain where cleavage occurs. Unfortunately, for series of glycans of different chain length, this number can change for the same fragmentation such as loss of the terminal residue from the reducing terminus. In order to overcome this difficulty we have introduced a modification whereby the subscript R is used for this cleavage and R-1 etc. is proposed for cleavages further into the glycan chain. For branched structures, chains are further designated by Greek letters with the largest chain being alpha. Ions retaining the charge at the nonreducing terminus are named similarly using A, B and C in place of X, Y and Z. Cross ring cleavages are further defined by superscript numbers preceding the letter and consisting of the lowest number of the two carbon atoms of the bond cleaved or "O" if the O-1 bond is cleaved. Major ions in positive ion spectra tend to be formed by B and Y glycosidic cleavages whereas ions in the negative ion spectra are mainly products of cross-ring or C-type glycosidic cleavages.

The high information content of the negative ion fragmentation spectra arises from abstraction of protons from individual and specific hydroxyl groups which catalyses specific electron movements leading to the diagnostic fragments as illustrated in **Fig. 3** for formation of the $^{2,4}A_R$ ions representing loss from the reducing terminal GlcNAc residue. Unfortunately, acidic glycans, such as those containing sialic acid, loose the acidic protons in preference to the hydroxylic hydrogens, thus suppressing formation of the diagnostic ions. Much specificity can be restored by blocking the acidic group by formation of methyl esters but the resulting fragmentation spectra are complicated by many additional ions formed by losses of methanol.

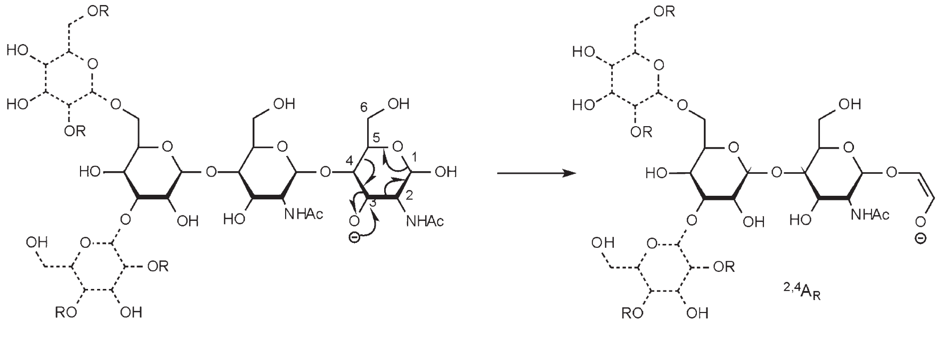

Fig. 3. Proposed mechanism for the formation of the $^{2,4}A_R$ ion from *N*-linked glycans.

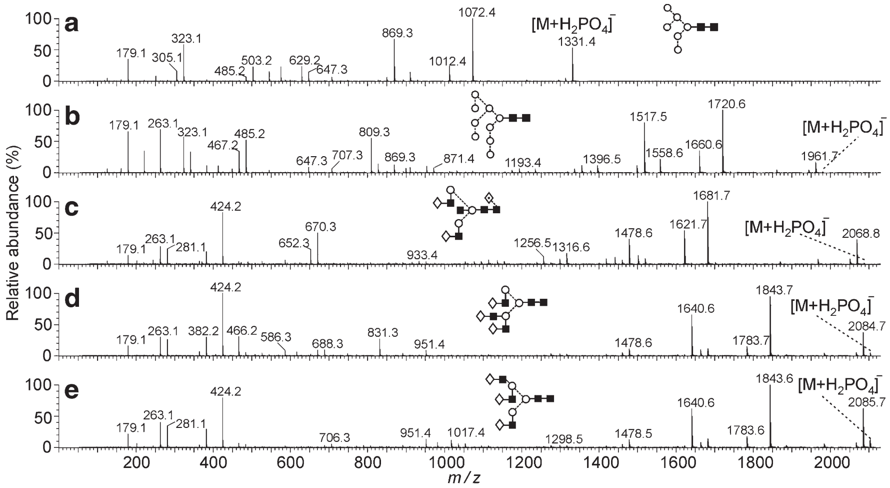

Fig. 4. Negative ion CID fragmentation spectra of (**a**) the high-mannose glycan $\mathrm{Man_5GlcNAc_2}$ (**b**) the high-mannose glycan $\mathrm{Man_9GlcNAc_2}$ (**c**) a fucosylated, bisected complex glycan (**d**) a 3-branched triantennary glycan (**e**) a 6-branched triantennary glycan.

A suggested spectral interpretation protocol could be as follows: typical spectra are shown in **Fig. 4**.

1. Determine the constituent monosaccharide composition of the glycans producing each peak using the information in **Table 1**. The mass of the glycans recorded by nano-electrospray as phosphate adducts will be 98 mass units higher than this calculated mass (mass of glycan + $H_2PO_4^-$) and those in the MALDI-TOF spectra will be 23 mass units higher ($[M+Na]^+$ ion).
2. From the negative ion MS/MS spectrum, verify that the adduct is phosphate. This can be done by locating the $^{2,4}A_R$, B_R and $^{2,4}A_{R-1}$ triplet at 259, 319 and 462 mass units below the mass of the molecular ion **(Fig. 4b)** or at 405, 465 and 608 mass units below if the glycan contains a fucose residue attached to the reducing terminal GlcNAc residue **(Fig. 4c)**. There is a possibility of other modifications and this must also be taken into account but most common *N*-linked glycans will fit this pattern. The most likely alternative adduct is chlorine which will add 35 and 37 mass units to the mass of the glycan and whose ions can often be identified by the characteristic chlorine isotope pattern.
3. Use the information in **Table 2** and the four publications ***(40–43)*** to deduce the structure of the glycan. Some of the more important diagnostic ions are outlined below:
4. The masses of the $^{2,4}A_R$, B_R and $^{2,4}A_{R-1}$ ions can be used to determine the presence of fucose at the 6-position of the reducing terminal GlcNAc residue; fucose at the 3-position will result in the absence of the $^{2,4}A_R$ ion because its formation involves abstraction of the hydrogen from this position in the initial loss of the adduct.

5. The D-type ions in **Table 2** (*m/z* 647 in **Fig. 4a**, 971 in **Fig. 4b**, 688 in **Fig. 4d**, and 1053 in **Fig. 4e**) consist of the β-linked core mannose residue together with the mannose attached to the 6-position together with its attached residues. Thus, they can be used to determine the composition of the 6-antenna and classify the glycan into complex or high-mannose/hybrid. The ion is accompanied by a second ion 18 mass units lower of comparable abundance. If a bisecting GlcNAc is present **(Fig. 4c)**, the D ion is absent because the bisecting GlcNAc residue is readily expelled (221 mass units) to give a very abundant ion at the same mass as the D-18 ion from the un-bisected glycans.
6. Antenna composition is revealed by a ^{1,3}A cleavage of the mannose residue in that antenna to produce what is normally a very abundant ion termed an F-type ion (e.g., antennae containing Gal-GlcNAc residues produce this ion at *m/z* 424 (Gal-GlcNAc + 59 mass units, *m/z* 424 in **Figs. 4c**, **4d**, and **4e**). If fucose is present on this antenna, the ion shifts to *m/z* 570. Shifts produced by other substituents are listed in **Table 2**.
7. An ^{O,4}A-cleavage of the antennae mannose residues from an unbranched antenna of composition Gal-GlcNAc gives an ion at *m/z* 466, an ion termed E. If the 3-antenna is branched **(Fig. 4d)**, an abundant E-type ion is present at *m/z* 831 (*m/z* 466 + the mass of Gal-GlcNAc). Branching of the 6-antenna **(Fig. 4e)** does not produce this shift because the 6-position, the substitution position of the second branch in the 6-antenna is not present in the E-ion. Thus, identification of the branching pattern of triantennary glycans is very simple ***(44)***.
8. The residues at the nonreducing termini of the antennae are revealed by the C_1 ion: *m/z* 179 when hexose is present or *m/z* 220 when chains terminate in GlcNAc.
9. Other diagnostic ions and masses are listed in **Table 2**.

Table 2
Ions Defining Structural Features in the Negative Ion Spectra of *N*-Linked Glycans

Structural feature	Ion	Ionic composition	*m/z*
Composition	Molecular	$[M+X]^-$	-
Antenna sequence	C	Gal, Man, Glc	179
		GlcNAc, GalNAc	220
		[Fuc]Gal	325
		Gal-[Fuc]GlcNAc	528
		αGal-Gal, Man-Man	341
		Man-[Man]Man	503
		GalNAc-GlcNAc	423
Antenna composition	F	Man	221
		GlcNAc	262
		Gal-GlcNAc	424
		Gal-[Fuc]GlcNAc	570
		αGal-Gal	586
		GalNAc-GlcNAc	465
		$(Gal-GlcNAc)_2$	789

(continued)

Table 2 (continued)

Structural feature	Ion	Ionic composition	*m/z*
		(Gal-GlcNAc)$_2$Fuc	935
		(Gal-GlcNAc)$_3$	1154
Fucose at 6-position of reducing terminus	$^{2,4}A_R$	[M-HCl-307]$^-$	[M-343]$^-$
		[M-HNO$_3$-307]$^-$	[M-370]$^-$
		[M-H$_3$PO$_4$-307]$^-$	[M-405]$^-$
Absence of fucose at 6-position of reducing terminus	$^{2,4}A_R$	[M-HCl-161]$^-$	[M-197]$^-$
		[M-HNO$_3$-161]$^-$	[M-224]$^-$
		[M-H$_3$PO$_4$-161]$^-$	[M-259]$^-$
Composition of 6-antenna	D and [D-18]$^-$ ([D-36]$^-$)	GlcNAc	526, 508
		Gal-GlcNAc	688, 670
		Gal-[Fuc]GlcNAc	834, 816
		(Gal-GlcNAc)$_2$	1053, 1035 (1017)
		(Gal-GlcNAc)$_2$Fuc	1199, 1181 (1163)
		Man$_3$	647, 629
		Man$_4$	809, 791
		Man$_5$	971, 953
	$^{O,3}A_{R-2}$ and $^{O,4}A_{R-2}$	GlcNAc	292, 262[1]
		GalGlcNAc	454, 424
		Gal-[Fuc]GlcNAc	600, 570
		(Gal-GlcNAc)$_2$	819, 789
		(Gal-GlcNAc)$_2$Fuc	965, 935
		Man$_3$	251, 221
		Man$_4$	413, 383
		Man$_5$	575, 545
Composition of 3-antenna	$^{O,4}A_{R-3}$ (E) ion	Gal-GlcNAc	466
		GlcNAc$_2$	507
		Gal-GlcNAc$_2$	669
		(Gal-GlcNAc)$_2$	831
		(Gal-GlcNAc)$_2$-Fuc	977
Presence of bisect	Abundant [D-221]$^-$ ion. Ion at [M-221-18]$^-$ Missing Ion D	GlcNAc	508
		Gal-GlcNAc	670
		(Gal-GlcNAc)$_2$-Fuc	816
		(Gal-GlcNAc)$_2$	1035
		(Gal-GlcNAc)$_2$Fuc	1181
		Man$_3$	629
		Man$_4$	791
		Man$_5$	953
Presence of sialic acid	B$_1$	Neu5Ac	290
		Neu5Gc	306
Presence of α2→ 6-linked sialic acid	$^{O,4}A_2$-CO$_2$	Neu5Ac	306
		Neu5Gc	322

1, These ions are normally of relatively low abundance. An abundant ion at the mass of the $^{O,4}A$ ion is more likely to be an F-type ion.

4. Notes

1. Several other matrices are available (see ***[ref. 7]***) that claim to produce advantages for certain types of carbohydrate.
2. The conditioned membranes can be stored in water for several months without noticeable loss of activity. Once used, they can be regenerated by repeating the nitric acid treatment.
3. Solutions containing organic solvents should be avoided.
4. The drops should remain at the same volume but often they increase slightly as water is drawn through the membrane. If the samples contains glycerol (found in some PNGase F preparations), considerable amounts of water can be drawn into the sample and the analysis usually fails. PNGase preparations containing glycerol should, consequently, be avoided.
5. The dried sample usually has long needle-shaped crystals projecting from the periphery of the spot towards the center. If the deposit is sticky, then either the sample is much too concentrated or it contains a high concentration of detergent or glycerol. Such deposits are unlikely to yield good spectra and the sample should be purified further.
6. Some investigators prefer to premix the matrix and sample solutions prior to application to the MALDI target. In the author's hands, this method does not appear to offer any particular advantages.
7. Only ~0.2 μL ethanol is necessary, and care must be taken to prevent spreading the sample over a larger area. This procedure is used to produce a more homogeneous target than can be achieved when water is present.
8. Sialylated glycans frequently give abundant peaks in positive ion mode due to loss of sialic acid when their spectra are recorded with reflectron-TOF instruments. Consequently, their spectra are often recorded in linear mode in order to minimize observed sialic acid loss. However, it must be borne in mind that this technique only removes PSD ions and not those fragment ions produced within the ion source. Minimizing the in-source delay is the best method for reducing the abundance of these ions. Also, it must be borne in mind that the resulting mass measurement may well be of average rather than a monoisotopic mass because of the lower resolution.
9. This effect is less pronounced with delayed-extraction instruments, but increased laser power can still lead to reduced resolution.
10. The laser spot should be moved to different areas of the target during acquisition, because not all areas of the target will yield a signal. It is frequently found with DHB that an active spot will only give a signal for a few laser shots due either to depletion of the sample/matrix mixture or vertical inhomogeneities within the crystal. Recent work ***(45)*** has suggested

that carbohydrates reside predominantly on the outside of the crystals, accounting for this phenomenon.

11. Usuaully 60–70 laser shots will be sufficient but more will be required for weak signals.
12. The mass spectrometer should be calibrated. Suitable calibration compounds are a mixture of dextran oligomers (e.g. dextran from *Leuconostoc ssp*. from Fluka,) for positive ion mode (*m/z* 203.05, 365.11, 527.16, 689.21, 851.26, 1013.32, 1175.37, 1337.42, 1499.48, 1661.53, 1823.58, 1985.63, 2147.69, 2309.74, 2471.79, 2633.85, 2795.90, 2957.95, 3120.00, 3282.06, 3444.11, 3606.16, 3768.22, 3930.27, 4092.32; and the same compounds derivatized with 2-aminobenzoic acid for calibrating negative ion spectra (*m/z* 300.11, 462.16, 624.21, 786.27, 948.32, 1110.37, 1272.43, 1434.48, 1596.53, 1758.58, 1920.64, 2082.69, 2244.74, 2406.80, 2568.85, 2730.90, 2892.95, 3055.01, 3217.06, 3379.11, 3541.17, 3703.22, 3865.27, 4027.32).
13. Formation of methyl esters allows all compounds to be examined as neutral molecules in positive ion mode. Without methylation, as well as laser-induced decomposition of sialylated glycans, these compounds will form both positive and negative ions, thus splitting the signal and the sialic acids will give additional ions by formation of sodium and potassium salts.
14. Although negative ion spectra are usually associated with the formation of $[M-H]^-$ ions, these tend to be unstable and fragment in the mass spectrometer ion source. Addition of an inorganic salt such as ammonium chloride, nitrate or phosphate produces $[M+anion]^-$ ions that are much more stable and do not fragment until they reach the collision cell of the mass spectrometer. Phosphate is used because it has been found that phosphate adducts are the major species observed from glycans released from gel-separated samples. Fragmentation of adducts with different anions is virtually identical because the first loss is that of the adduct together with a proton to give what is effectively the $[M-H]^-$ ion. Larger anions such as bromide and iodide should be avoided because these produce very few fragment ions.
15. The tip should be long enough to reach to the end of the capillary.
16. MS/MS spectra of major components can be obtained in a few seconds but those of weaker components can be accumulated for many minutes in order to achieve a satisfactory signal:noise ratio. The spray should last on average from 1–3 hours.
17. It is assumed that the mass spectrometer has been set up and is operated according to the manufacturer’s instructions. Suitable calibration compounds for negative ion work are the 2-AA-derivatized glycans listed above or underivatized glycans released from bovine fetuin.

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MS Analysis of Protein Glycosylation

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1. Introduction

Post-translational modifications (PTM) are important molecular events because they generate functional diversities of proteins. A large number of proteins undergo multiple PTM *in vivo* and over 300 types of PTM have been identified to date ***(1)***. Among various PTM, phosphorylation and glycosylation have been intensely studied because of their physiological significance. Phosphorylation plays key regulatory roles in cellular signaling processes ***(2)***, and glycosylation mediates crucial cellular mechanisms such as protein folding and trafficking ***(3, 4)***. To confirm the presence of these modifications *in vivo*, the conventional analytical procedures require large quantities of samples and laborious biochemical steps. For this reason, small-scale analysis of PTM remains among the most challenging areas of biological science.

This chapter describes our recent proteomic methodology using the combination of two-dimensional gel electrophoresis (2-DGE) and MALDI quadrupole ion trap time-of-flight mass spectrometer (MALDI-QIT-TOF MS), which enables a highly sensitive analysis of post-translational modifications (PTM). Two-DGE has been used extensively as a powerful tool to separate proteins from a limited amount of biological samples ***(5–8)***. We stained gels with Pro-Q™ Emerald/Diamond dyes immediately after 2-DGE and prior to CBB staining, and because this allows us to perform direct detection of glycoproteins/phosphoproteins on the same 2-D gels ***(9,10)***. Gel-separated glycoproteins are further subjected to the structural analysis using MALDI-QIT-TOF MS. MALDI-QIT-TOF MS is capable of structural characterization of various PTMs through highly sensitive fragmentation, or MS/MS, analysis ***(11,12)***. In MALDI-QIT-TOF MS, two mass analyzers (i.e., QIT and TOF) are connected to MALDI ion source, and the combination of MALDI and QIT renders a unique opportunity to perform multistage fragmentation (MS^n) analysis with high-energy collision-induced

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dissociation (CID) ***(11,13)***. In this chapter, we will describe proteomic techniques that we use to analyze *N*-linked glycosylation. However, the protocol can be applied to other PTM analyses with some modifications.

2. Materials

1. MALDI-QIT-TOF MS (AXIMA QIT; Shimadzu Biotech, Manchester, UK)
2. MALDI-TOF MS
3. MALDI stainless sample target
4. Acetonitrile (HPLC grade)
5. Ammonium bicarbonate
6. Trifluoroacetic acid (TFA)
7. Milli-Q water (Millipore, Bedford, MA, USA)
8. Sequencing-grade trypsin (Promega Madison, WI, USA).
9. Stock solutions for in-gel digestion:
    a. 50% (v/v) acetonitrile/100 mM ammonium bicarbonate (pH 8.9)
    b. 50% (v/v) acetonitrile/5% (v/v) TFA
    c. 0.2% (v/v) TFA solution
    d. 10 mM ammonium bicarbonate (pH 8.9)
10. Recrystalized 2,5 dihydroxybenzoic acid (DHB)
11. DHB solution for MS analysis: 2% (w/v) DHB in 50% (v/v) acetonitrile/0.1% (v/v) TFA (*see* **Note 1**)

3. Methods

Figure 1 shows a schematic diagram of our glycoprotein analysis. Protein samples are first separated using 2-DGE, and glycoprotein spots are detected using a glycoprotein staining reagent **(Subheading 3.1)**. Glycoprotein spots are in-gel digested with trypsin **(Subheading 3.2)** (*see* **Note 2**), and identified by peptide mass fingerprinting analysis **(Subheading 3.3)**. In order to characterize their glycan structures and modification sites, tryptic glycopeptides are further subjected to fragmentation analysis in MALDI-QIT-TOF MS **(Subheading 3.4)**. Because human keratin contamination interferes with MS analysis, it is recommended that powder-free latex gloves be worn throughout the procedure.

3.1. Gel Electrophoresis and Glycoprotein Staining

1. Perform 2-DGE to resolve glycoproteins. We perform isoelectric focusing for the 1st dimension and SDS-PAGE for the 2nd dimension (*see* **Note 3**). Instead of 2-DGE, one-dimensional gel electrophoresis (e.g., SDS-PAGE) can be used for this protocol. However, the resolution of proteins on a one-dimensional gel is generally inadequate for the separation of complex protein mixtures.
2. Following 2-DGE, gels are stained with a glycoprotein staining reagent for detection of glycoproteins (*see* **Note 4**) or followed by Coomassie Brilliant Blue (CBB) for visualization of all protein spots (see example in **Fig. 2**).

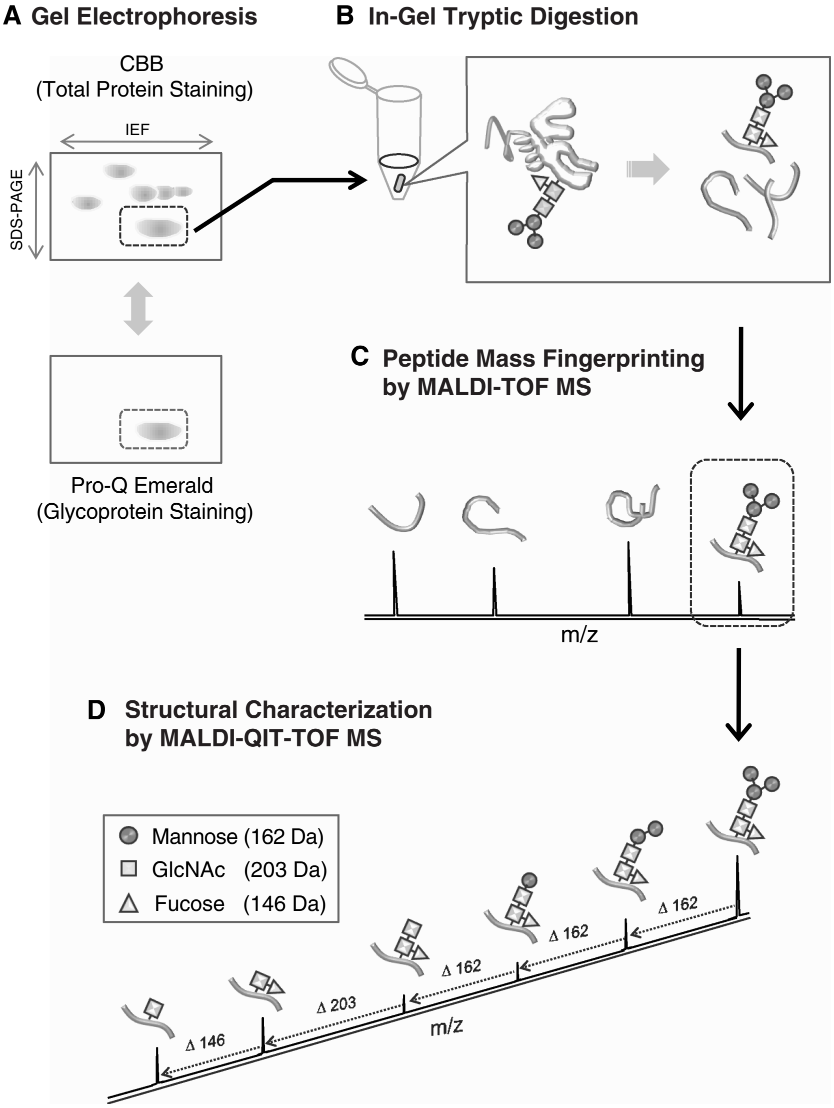
A Gel Electrophoresis
B In-Gel Tryptic Digestion
CBB
(Total Protein Staining)
IEF
SDS-PAGE
Pro-Q Emerald
(Glycoprotein Staining)
C Peptide Mass Fingerprinting by MALDI-TOF MS
m/z
D Structural Characterization by MALDI-QIT-TOF MS
● Mannose (162 Da)
□ GlcNAc (203 Da)
△ Fucose (146 Da)
Δ 146
Δ 203
Δ 162
Δ 162
Δ 162
m/z

Fig. 1. A schematic drawing of glycoprotein analysis using 2-DGE and MALDI-QIT-TOF MS. **(A)** Proteins are separated by 2-DGE based on their isoelectric focusing points and molecular weights in the first and second dimension, respectively. A glycosylated protein spots are detected by staining with Pro-Q Emerald dye. **(B)** After in-gel digestion of the glycoprotein spot with trypsin, the tryptic peptides are subjected to peptide mass fingerprinting analysis. **(C)** Peptide mass fingerprinting analysis provides the mass profiles and sequence information of the tryptic peptides. **(D)** A glycopeptide thus detected is further fragmented by MALDI-QIT-TOF MS. Using the information from fragment ions, the structure of a glycan moiety can be deduced.

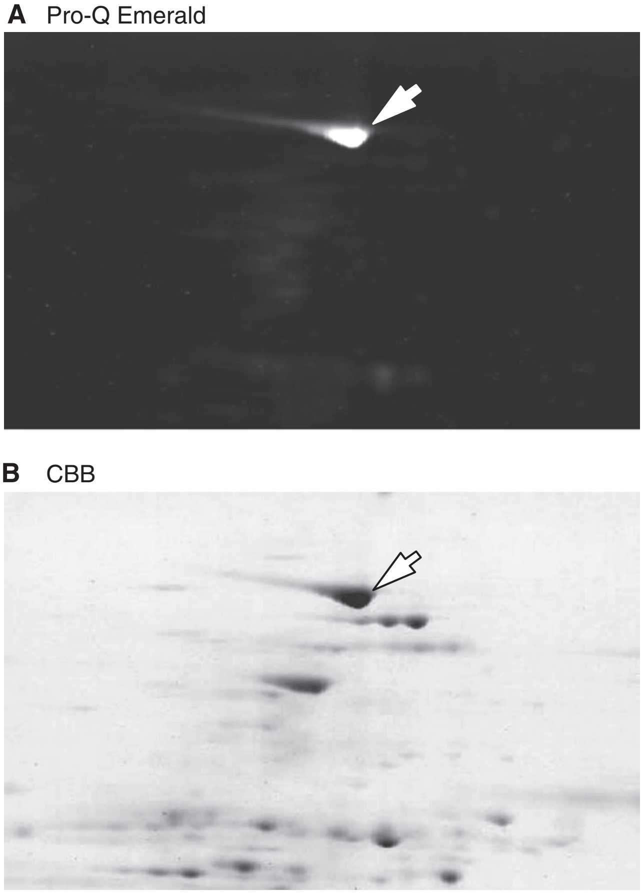

Fig. 2. Differential staining of *Drosophila* proteins with a fluorescent glycosylation sensor dye, Pro-Q™ Emerald (**A**) and Coomassie blue (**B**). *Drosophila* eye proteins were separated on 2-D gel. Glycosylated protein spot *(arrow)* was identified by staining with Pro-Q Emerald dye. Our peptide mass fingerprinting analysis identified the spot as chaoptin.

3.2. In-Gel Tryptic Digestion

1. After detection of protein spots positive for glycoprotein staining, corresponding protein spots stained with CBB are excised by a razor blade and transferred into a 1.5 ml polypropylen tube. Excised gel pieces are shaken in 1 ml of 50% (v/v) acetonitrile/100 mM ammonium bicarbonate (pH 8.9) at room temperature to remove CBB.
2. Distained gel pieces are incubated with 1 ml of acetonitrile for 30 min to dehydrate the gels. After removing acetonitrile, the gel pieces are rehydrated with 2 µl of trypsin solution for 30 min at 4°C. Add 50 µl of 10 mM ammonium bicarbonate (pH 8.9) and incubate at 37°C for 5–10 hrs. During the incubation, trypsin continues

to digest a protein in a gel piece and while some of the tryptic peptides come out of the gel piece.

3. Transfer the solution containing tryptic digests into a new 0.65 ml polypropylen tube. To extract the rest of the tryptic digests from the gel, vigorously shake the gel in 50 µl of 50% (v/v) acetonitrile/5% (v/v) TFA for 20 min. After a brief centrifuge, collect and transfer the supernatant into the new tube to mix with the original digest solution.
4. Concentrate the collected peptide solution down to near dryness using vacuum centrifuge (*see* **Note 5**).

3.3. Peptide Mass Fingerprinting Analysis using MALDI-TOF MS

1. Each peptide sample is reconstructed with 5 µl of 0.2% (v/v) TFA solution. Load the peptide solution (0.5 µl) onto a MALDI-TOF sample target plate with a fresh DHB solution (0.5 µl) and dry the mixture completely at room temperature.
2. Peptide samples on the MALDI sample target are subjected to peptide mass fingerprinting analysis using MALDI-TOF MS. After measuring the masses of the tryptic peptides by MALDI-TOF MS, the observed masses are submitted to MASCOT PMF search program (Matrix Science, London, UK) at http://matrixscience.com.
3. We routinely perform MASCOT search against the latest version of the National Center for Biotechnology Information (NCBI) nonredundant database using the following parameters; (a) unlimited protein molecular weight and pI ranges, (b) presence of protein modifications including acrylamide modification of cysteine, methionine oxidation, protein N-terminus acetylation, and pyroglutamic acid, and (c) mass tolerance of ± 0.25 Da. We consider the confirmation to be positive when a significant MOWSE score ($p < 0.05$) is generated.
4. After the protein identification by peptide mass fingerprinting analysis, the masses of the observed peptides are compared to that of the theoretical tryptic peptides. The peptides which contain glycans will be detected as the unassigned ion peaks because glycosylation leads to a predictable increase in the mass of a modified amino acid residue. When unassigned ion peaks are detected in MS spectrum, the masses of the unassigned peaks are subjected to ExPASy-GlycoMod Tool (http://au.expasy.org/tools/glycomod) to predict possible glycan structures and modification sites. To calculate the theoretical fragment ions resulting from CID fragmentation of the predicted peptide, we use MS-Product program (http://prospector.ucsf.edu/).

3.4. Structural Characterization of Protein Glycosylation Using MALDI-QIT-TOF MS

1. Load peptide solution (0.5 µl) onto a MALDI-QIT-TOF sample target with a fresh DHB solution (0.5 µl) and dry the mixture completely at room temperature. Each peptide sample is subjected to MALDI-QIT-TOF MS analysis.
2. In general, an ionized glycopeptide is fragmented without CID in MALDI-QIT-TOF MS, and as a result, a precursor glycopeptide together with some of its fragment ions will be detected in MS mode. Sugar components are readily

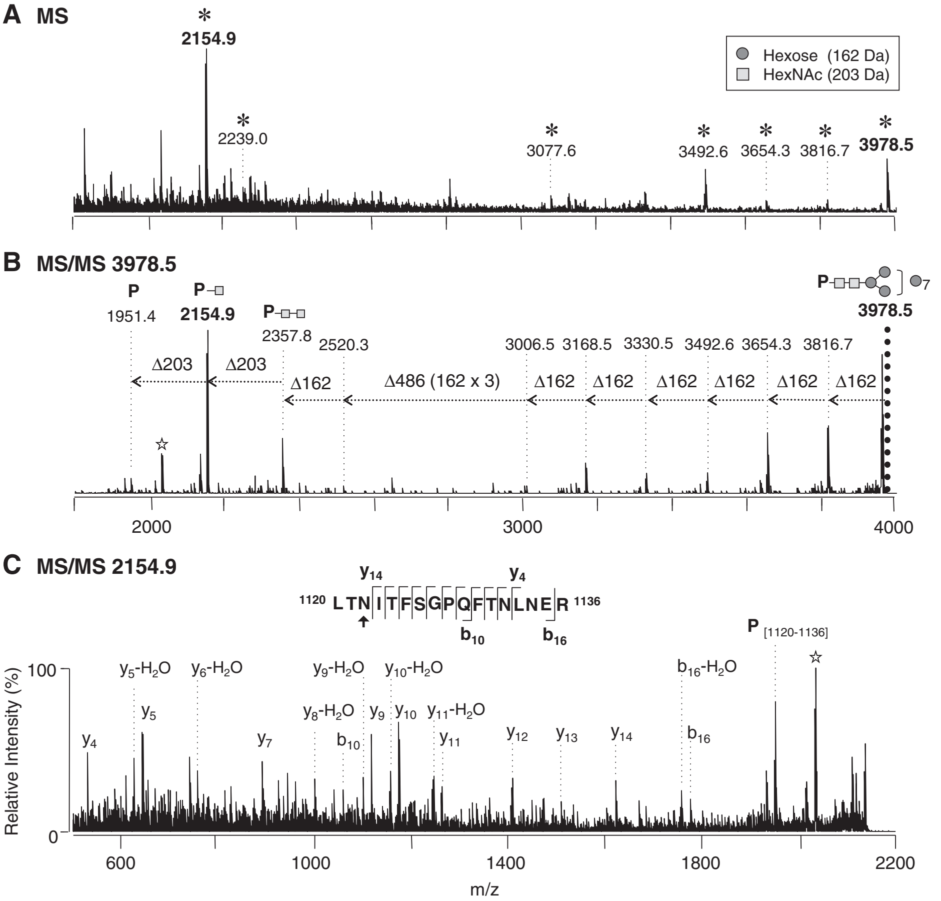

Fig. 3. Structural characterization of chaoptin glycopeptide using MALDI-QIT-TOF MS. The tryptic peptides of chaoptin (*Drosophila* glycoprotein) separated on 2-D gel (**Fig. 2**) were subjected to a series of CID fragmentation to deduce the glycan sequence and glycosylation site. **(A)** MS spectrum of the tryptic digests. *, peptide ions not assigned as tryptic peptides of chaoptin. **(B)** MS/MS spectrum of an unassigned ion peak at m/z 3978.5 observed in MS mode. CID fragmentation resulted in a series of fragment ions separated by 162, 203, or 486 (162 × 3) Da. **(C)** MS/MS spectrum of a fragment ion at m/z 2154.9. The y ion and b ion series derived from the peptide corresponded to the amino acid residues 1120 to 1136 of chaoptin. ↑, a putative *N*-linked glycosylation site. ☆, a peptide ion with a cross-ring cleavage of HexNAc residue. ○ in dark gray, hexose residue; □ in light gray, *N*-acetylhexosamine (HexNAc) residue. (With permission from ***ref. 11***)

distinguishable from amino acid residues and other post-translationally modified residues based on their masses. As a result, we can easily distinguish glycopeptides from other nonglycopeptides.

3. To determine the actual glycan structure, an ionized glycopeptide is further subjected to fragmentation analysis in MS/MS mode. As compared with peptide-bonds, glycosidic bonds are susceptible to CID fragmentation resulting in a sequential loss of sugar components from the terminal end of a glycan moiety (see example in **Fig. 3**).
4. Because some peptide-bonds seldom undergo total dissociation in a single run of MS/MS analysis, further fragmentation analysis by MS^n analysis may be required to determine the amino acid sequence and modification site(s).

4. Notes

1. Prepare fresh DHB solution just before use.
2. Some glycoproteins are not susceptible to tryptic cleavage due to the sterical hindrance of their glycans. Digestion with other proteases may improve the recovery of glycopeptides.
3. In our laboratory, isoelectric focusing gel electrophoresis is carried out using IPGphor (GE Healthcare/Amersham, Buckinghamshire, UK) with a cup loading strip holder. Immobiline Dry Strip (pH 3–10, 13 cm in length) is rehydrated for 15 hr at room temperature with 250 µl of lysis solution (8.5 M urea, 2% (w/v) CHAPS, 0.2% (w/v) Bio-Lyte 3/10 (BIO-RAD, Hercules, CA, USA), and 5% (v/v) β-mercaptoethanol). The same lysis solution (100 µl) is used for sample homogenization. After centrifugation at 15,000 × *g* for 10 min, the supernatant is loaded into a sample cup. Electrophoresis is performed at 500 V for 3 min, 4,000 V for 2 hr, and 8,000 V up to a total of 22,000 Vhr.
4. Several glycoprotein staining reagents are commercially available. We use Pro-Q™ Emerald glycoprotein gel stain (Molecular Probes, Eugene, OR, USA) because of its high sensitivity. Regarding the details of the Pro-Q staining, refer to manufacture's instructions.
5. Peptide samples can be stored at –20°C at least for several months.

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Mapping protein N-Glycosylation by COFRADIC

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1. Introduction

The majority of proteins require co- and posttranslational modifications to become functional entities. Such modifications fulfill a plethora of tasks (e.g., modulation of enzyme activity and directing proteins to cellular compartments).

One of these protein modifications that, amongst many others, plays an essential role in determining a protein's structure and functionality is N-glycosylation, which is the enzymatic attachment of a glycan to an asparagine in the consensus motif NXS/T/C ***(1,2)***. Next to asparagines, glycosylation can also occur on serine and threonine (O-glycosylation) ***(3–6)*** and tryptophan (C-glycosylation) ***(7)***. Protein N-glycosylation has intrinsic as well as extrinsic functions. Intrinsic functions brought about by this modification are the creation of structural modules and changes of the solubility, antigenicity and stability of the affected protein. Examples of extrinsic functions are the routing of proteins to a specific subcellular localization, and the guiding and modulation of cell adhesion and intracellular communication.

Congenital Disorders of Glycosylation (CDG) are a group of diseases caused by glycan synthesis errors ***(8)***. Several CDG subtypes are distinguished based on the malfunctioning of a specific enzyme in the protein-N-glycosylation pathway. Other states such as the malignant transformation of cells to cancerous cells portray altered glycan chains on glycoproteins ***(9–14)***. Both examples show the importance of protein glycosylation in the normal physiology and pathophysiology of organisms and illustrate the value of monitoring protein N-glycosylation sites on a proteomic scale.

Several methods for studying protein N-glycosylation have been described. Lectin based methods rely on the affinity of lectins for subsets of glycans to isolate glycosylated proteins or peptides ***(15,16)***. However, to identify the broadest possible range of N-glycosylated molecules, different lectins must be combined,

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either in parallel or serial, to a multilectin affinity column ***(14,17–20)***. Hydrazide based techniques chemically enrich N-glycosylated peptides and are not biased towards any specific glycan structure ***(21–23)***. Although the hydrazide technology offers a straightforward analysis of N-glycosylation sites, all information about the actual nature of the N-glycan is lost.

We recently developed a gel-free technique for analyzing protein-N-glycosylation ***(24)*** based upon combined fractional diagonal chromatography (COFRADIC ***25***) and we here describe a detailed protocol for isolating N-glycosylated peptides for MS/MS analysis. Briefly, proteins in a complex sample such as serum are digested with trypsin. These peptides are separated a first time on a RP-HPLC column (*primary COFRADIC run*) and time-based collected into several primary fractions. Sufficiently spaced primary fractions are combined, treated with PNGase F and reseparated by the same RP-HPLC setup (*secondary COFRADIC runs*). PNGase F cleaves N-glycans from asparagines and hereby converts these to aspartic acid, thus creating a marker (1 Da difference) that can be used to interpret results of database searches using MS/MS-spectra. Deglycosylated peptides display different retention on the reverse-phase column during the secondary HPLC separations and thus shift away from nonglycosylated peptides. These shifts finally lead to the specific collection of formerly in vivo N-glycosylated peptides which may then be analyzed by MS techniques.

2. Materials

2.1. Protein Preparation

1. Tris(2-carboxyethyl)phosphine (TCEP, Pierce, Rockford, IL).
2. Iodoacetamide (IA).
3. Hydrogen peroxide:(30% (w/w) in water.
4. Disposable desalting columns packed with Sephadex™ G-25 (GE Healthcare Bio-Sciences, Uppsala, Sweden).
5. Sequencing grade modified trypsin (Promega, Madison, WI, USA).

2.2. COFRADIC Protocol for the Isolation of N-Glycopeptides

1. Analytical RP-HPLC column: 2.1 mm internal diameter (I.D.) × 150 mm (length) 300SB-C18 column, Zorbax® (Agilent, Waldbronn, Germany).
2. HPLC grade water and acetonitrile.
3. PNGase F from *Elizabethkingia (Chrysobacterium/Flavobacterium) meningoseptіcum* (Sigma-Aldrich).
4. Acetic acid glacial, analytical reagent grade.

3. Methods

The methods described below use a serum proteome for characterization of N-glycosylation sites. It should be clear that any proteome or peptidome in which N-glycosylated asparagines are present can be sampled by these methods.

3.1. Preparation of Serum Proteins Prior to COFRADIC

1. 1 mL of mouse serum proteins (*see* **Note 1**) deprived of its three most abundant proteins is desalted on a NAP-10 column and eluted in 1.5 mL of 2 M guanidinium hydrochloride in 50 m*M* Tris-HCl pH 8.6.
2. Reduce the sample volume to 1 mL by vacuum drying.
3. Add freshly prepared TCEP.HCl and iodoacetamide solutions to 10 and 20 m*M* final concentrations respectively. Let the reduction and alkylation of cysteines proceed in the dark for 1 h at 37°C (*see* **Note 2**).
4. Desalt the mixture of modified proteins in 1.5 mL of freshly prepared 20 m*M* NH_4HCO_3 (pH 7.6) using a NAP-10 column.
5. Heat the protein mixture for 10 min at 95°C followed by cooling for 10 min on ice.
6. Add sequence grade modified trypsin (the enzyme/substrate ratio should be 1/50 to 1/100 (w/w)) and incubate overnight at 37°C.
7. Reduce the volume to 500 µL by vacuum drying.

3.2. COFRADIC Sorting of N-Glycosylated Peptides

1. Acidify 98 µL of the peptide mixture with 2 µL of a 50% (w/v) acetic acid solution (f.c. of 1%).
2. Add 2 µL of a 30% (w/v) hydrogen peroxide solution (f.c. of about 0.6% of H_2O_2) to oxidize methionines to sulfoxides. The peptides are incubated for 30 min at 30°C (*see* **Note 3**).
3. Load the sample immediately on the reverse-phase column (*see* **3.3**) for the primary COFRADIC separation and collect peptides in 56 consecutive fractions of 1 min each starting 25 min after sample injection (*see* **Note 4**).
4. Pool primary fractions that are spaced by 14 min and dry them under vacuum.
5. Redissolve the dried peptides in 97 µL of 50 mM NH_4HCO_3 (pH 7.6) and add 1 µL of a 0.5 U/µL stock solution of PNGase F. This mixture is incubated for 3 hours at 37°C to fully deglycosylate the N-glycosylated peptides.
6. Add 2 µL of a 50% (w/v) acetic acid solution and reseparate each PNGase F treated pool of primary fractions on the same RP-HPLC column under the same conditions. Per primary fraction, deglycosylated peptides are collected in two intervals: one 11 to 3 min prior to and one 2 to 10 min following the original collection interval. Thus, per secondary run, 5 such secondary intervals are collected, each consisting of 4 secondary fractions of 2 min (or 160 µL) (**Fig. 1** and **Table 1**).
7. Such isolated secondary fractions are dried and stored at −20°C until further MS/MS-analysis (*see* **Note 5**).

3.3. Reverse Phase HPLC Separation in COFRADIC

The solvent gradient described below is applied both for the primary and the secondary runs. We found it important to use a HPLC solvent system buffering around a pH of about 5. In contrast to lower pH values, at pH 5 the extent of the chromatographic shift of deglycosylated versus glycosylated peptides is primarily based on the removal or gain of charges. Due to the nature of the glycans, both hydrophilic and hydrophobic shifts can be expected. When the

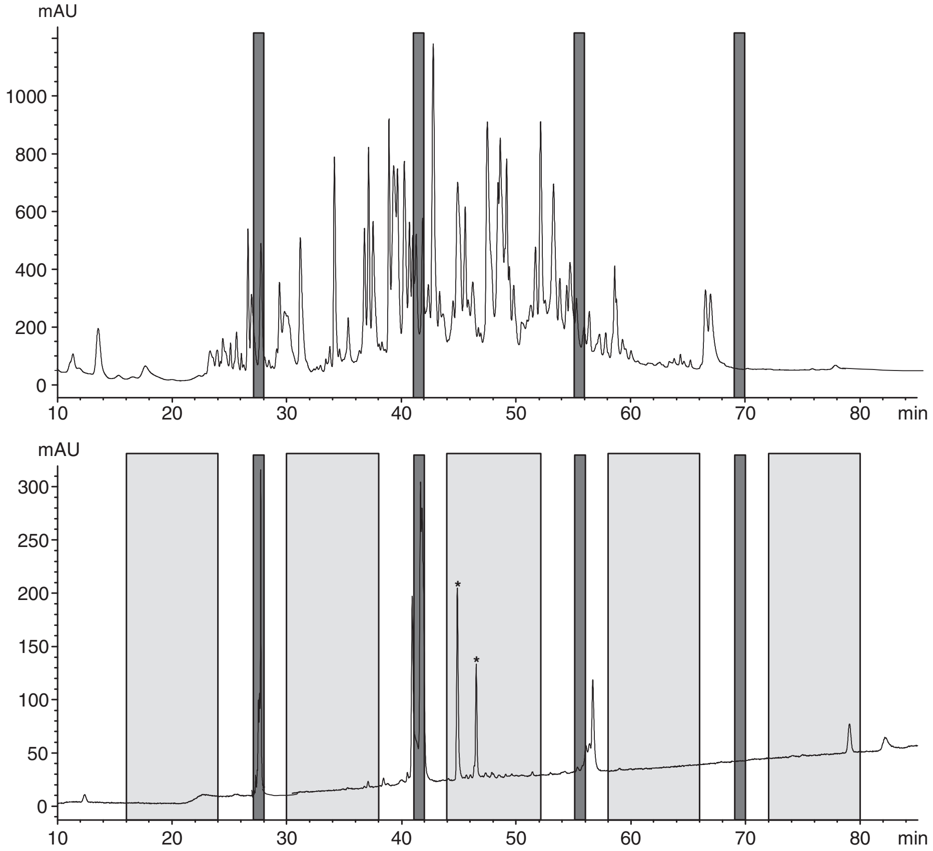

Fig. 1. Example of a primary and a secondary COFRADIC RP-HPLC run for isolating N-glycopeptides. The upper panel shows the primary RP-HPLC separation (UV absorbance at 214 nm) of a tryptic digest of human alpha-1-acid-glycoprotein and bovine fetuin; primary fractions were collected between 25 and 81 min and pooled as described in **Subheading 3.2**. Such collected peptides were then treated with PNGase F to remove N-linked glycan chains. The lower panel shows the secondary RP-HPLC separation of the PNGase F treated primary fractions that eluted between 27–28, 41–42, 55–56 and 69–70 min (indicated in dark grey boxes). The secondary collection intervals containing the shifted, deglycosylated peptides (indicated in light grey boxes) were collected 11 to 3 min prior to and 2 to 10 min following each primary fraction. There is a clear shift of peptides (indicated with an asterisk) that elute in the secondary collection interval between the primary fractions collected at 41–42 and 55–56 min. Further MS and MS/MS analyses identified tryptic peptides carrying known N-glycosylation sites of bovine fetuin (N99 and N176) and human alpha1-acid glycoprotein (N93).

Table 1
Fraction Pooling Scheme for COFRADIC Isolation of N-Glycosylated Peptides

One example of the pooling of secondary fractions is given (*see* Figure 1) in which it is made clear that peptides undergoing a hydrophobic shift following PNGase F treatment of fraction X overlap with fractions undergoing a hydrophilic shift from fraction X + 14 following the same treatment.

POOLING SCHEME OF PRIMARY FRACTIONS

	prim. fraction	elution interval	prim. fraction	elution interval	prim. fraction	elution interval	prim. fraction	elution interval
A	1	25–26 min	15	39–40 min	29	53–54 min	43	67–68 min
B	2	26–27 min	16	40–41 min	30	54–55 min	44	68–69 min
C	3	27–28 min	17	41–42 min	31	55–56 min	45	69–70 min
D	4	28–29 min	18	42–43 min	32	56–57 min	46	70–71 min
E	5	29–30 min	19	43–44 min	33	57–58 min	47	71–72 min
F	6	30–31 min	20	44–45 min	34	58–59 min	48	72–73 min
G	7	31–32 min	21	45–46 min	35	59–60 min	49	73–74 min
H	8	32–33 min	22	46–47 min	36	60–61 min	50	74–75 min
I	9	33–34 min	23	47–48 min	37	61–62 min	51	75–76 min
J	10	34–35 min	24	48–49 min	38	62–63 min	52	76–77 min
K	11	35–36 min	25	49–50 min	39	63–64 min	53	77–78 min
L	12	36–37 min	26	50–51 min	40	64–65 min	54	78–79 min
M	13	37–38 min	27	51–52 min	41	65–66 min	55	79–80 min
N	14	38–39 min	28	52–53 min	42	66–67 min	56	80–81 min

POOLING SCHEME OF SECONDARY FRACTIONS FROM PRIMARY POOL C (*cfr. Figure 1*)

	fraction 3	fraction 17	fraction 31	fraction 45	
C	16–24 min	30–38 min	44–52 min	58–66 min	72–80 min

glycan is not charged, the evoked shift will be hydrophilic by the deamidation of the asparagine to aspartic acid (gain of negative charge). If the glycan was charged (e.g., sialic acid, phosphate …) the glycopeptide looses charged residues and undergoes a hydrophobic shift. Lowering the pH of the solvent system (e.g., using TFA instead of ammonium acetate) will have minor effect on acidic residues (these will mainly be neutral) and was found to lead to insufficient chromatographic shifts and inefficient collection of deglycosylated peptides.

1. Prepare HPLC solvent A (10 m*M* ammonium acetate (*see* **Note 6**) (pH 5.5) in water/acetonitrile, 98/2 (v/v)) and HPLC solvent B (10 mM ammonium acetate (pH 5.5) in water/acetonitrile, 30/70 (v/v)).
2. The following RP-HPLC solvent gradient is applied:
    a. Following injection of the sample onto the column, apply a 10 min isocratic separation with 100% of solvent A at a constant flow rate of 80 µl/min.
    b. Apply a linear gradient to 100 % over 100 min (i.e. an increase of 1 % solvent B per minute).

c. Apply a 10 min isocratic wash with 100% of solvent B, followed by a linear gradient over 5 min to 0% of solvent B (100% of solvent A).

d. Reequilibrate the column for another 20 min with 100% of solvent A before injection of another sample.

4. Notes

1. For the studies mentioned above, we were specifically interested in identifying low-abundant N-glycosylated serum proteins. Therefore, we performed MARS (multiple affinity removal system) depletion of abundant serum proteins (albumin, transferrin and IgG's) to increase the possibility of identifying low-abundant glycoproteins (26). MARS depletion (Agilent, Waldbronn, Germany, #5188–5218) was applied to 90 µL serum from a male C57 black strain mouse according to the manufacturer's protocol and yielded a protein mixture with a concentration of about 1 mg/mL. We then typically load a peptide amount equivalent to about 200 µg of protein material on the RP-column for the primary COFRADIC run.
2. Blocking cysteine residues by iodoacetamide avoids the formation of disulfide bridges between different cysteinyl peptides further on in the experiment. For the alkylation of cysteines we use an estimated 750-fold molar excess of TCEP and a 1500-fold molar excess of IA over the proteins (an average molecular weight of 60,000 g/mol for proteins is considered here). Addition of TCEP-HCl can change the pH of the protein solution and it is therefore necessary to check the pH and correct it by adding the appropriate volumes of a 1 M stock solution of NaOH. It is crucial to keep the pH at around 8 since a more acidic pH may result in an incomplete alkylation.
3. Oxidation of methionines by hydrogen peroxide prior to the primary COFRADIC run prevents unwanted shifts of methionine peptides during the secondary runs. Spontaneous oxidation of methiones between the two runs will cause a hydrophilic shift of nonglycosylated methionyl peptides *(25)* that will finally get sampled by the mass spectrometers. Methionine oxidation should be carried out strictly as described in **Subheading 3.2.2** since prolonged incubation lead to nonquantitative formation of methionine-sulphon, oxidation of tryptophan and cysteine.
4. Most of the peptides separated using the described RP-HPLC gradient and solvents will elute between 25 min and 81 min. Intuitively, depending on the peptide elution profile during the primary run, the collection scheme should be adapted.
5. Following COFRADIC, N-glycosylated peptides are characterized by deamidation of deglycosylated asparagines in NXS/T motif. However, spontaneous deamidation between the primary and secondary runs (especially at Asn-Gly and Asn-Ser sites *(27)*) also leads to hydrophilic shifts of non-glycosylated peptides into the secondary collection intervals.

However, such peptides will be identified with a deamidated asparagine that resides outside the NXS/T motif and should be filtered out of the final list of identifications.

6. For the ammonium acetate buffer system we use a 500 mM ammonium acetate stock solution that is prepared as follows: for 2 L of this stock solution we titrate 57.19 mL of acetic acid with NH_4OH to a pH of 5.5 (about 60 mL of NH_4OH should be added).

Acknowledgments

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Mass Spectrometric Analysis of *O*-Linked Glycans Released Directly from Glycoproteins in Gels Using β-Elimination

Adrian M. Taylor and Jane Thomas-Oates

1. Introduction

Extensive posttranslational modification of proteins through the glycosidic attachment of carbohydrate moieties to the backbone of polypeptides makes glycosylation an important area of study. The function of these glycan chains may be to affect the conformation and stability of a protein ***(1)***, or to perform essential roles in cell surface or intracellular recognition and cell adhesion and therefore play important roles in mediating biological activity ***(2)***.

Analysis of glycoproteins is made difficult by the sheer structural diversity of glycans attached to proteins, and because each glycosylated polypeptide is generally associated with a population of different glycan structures frequently attached at more than one site, possibly via more than one different type of chemical linkage. Consequently, detailed structural analysis of one or more glycans is impractical while the glycans are still attached to the polypeptide; release of the glycan is therefore required. Traditional methods used for glycan release employ both chemical and enzymatic approaches. The most commonly used chemical methods for the release of *O*-linked glycans are based on β-elimination in solution. Once the glycans have been released from the protein, methods for the analysis of glycoprotein glycans generally require a combination of techniques that include mass spectrometry, nuclear magnetic resonance (NMR) spectroscopy, monosaccharide compositional analysis and linkage analysis.

Protein characterisation has been revolutionised by proteomics approaches, in which mixtures of proteins and glycoproteins are routinely separated using SDS-PAGE. The need to identify not only the polypeptide but also its posttranslational

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modifications has driven a whole range of method developments aimed at identifying posttranslational modifications on proteins that have been separated by gel electrophoresis.

We, and others, have described blotting glycoproteins from gels onto polyvinylidene fluoride (PVDF) membranes prior to either PNGase F release of *N*-linked glycans ***(3,4)*** or reductive β-eliminative release of *O*-linked glycans ***(5,6)***, and one paper has described the success of directly performing in-gel protein de-*N*-glycosylation using PNGase F ***(7)***. Kilz and coworkers have described an analogous in-gel HF-pyridine de-*O*-glycosylation ***(8)***. However, such reagents are toxic, so that alternative in-gel protein de-*O*-glycosylation methods were sought. One such procedure, a modification of the nonreductive base-catalysed β-elimination method developed by Rademaker *et al.* ***(9)***, has been used for in-gel de-*O*-glycosylation/ethylaminylation ***(10)***, but was employed for glycoprotein identification and localisation of *O*-glycosylation sites; no attempt was made to recover the released glycans for further analysis.

In this chapter we detail a method ***(11)*** for reductive β-eliminative release of the intact *O*-linked glycans from SDS-PAGE separated glycoproteins, and their mass spectrometric analysis. The method leaves the deglycosylated polypeptide in the gel for identification using established proteomic approaches, based on in-gel tryptic digestion and mass spectrometric analysis of the released peptides.

2. Materials

2.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Resolving buffer: 0.5 M Tris-HCl, pH 6.8, 0.1% SDS. Store at room temperature.
2. Stacking buffer: 0.5 M Tris ((hydroxymethyl) methylamine)-HCl, pH 6.8, 0.1% SDS. Store at room temperature.
3. 30% degassed acrylamide/Bis (N,N-bis-methylene-acrylamide) (30% T, 2.67% C), where for acrylamide gels:

   %T = {g (acrylamide + bisacrylamide)/100 mL} 100
   %C = {g (bisacrylamide)/g (acrylamide + bisacrylamide)} 100

This acrylamide solution is a neurotoxin when unpolymerised and precautions should be taken to limit direct exposure.

4. TEMED (N,N,N,N-tetra-methyl-ethylenediamine), best stored at room temperature in a desiccator.
5. Ammonium persulfate: Prepare 10% solutions in water and immediately freeze in aliquots appropriate for single use at −20°C.
6. Sample Buffer: DDI H_2O (3.55 mL), 0.5 M Tris-HCl, pH 6.8 (1.25 mL), glycerol (2.5 mL), 10% w/v SDS (2.0 mL), 0.5% (w/v) bromophenol blue (0.2 mL):

Total volume 9.5 ml. Add 50 μL β-mercaptoethanol to 950 μL sample buffer prior to its use.

7. 10x Electrode (Running) Buffer, pH 8.3 (1 L) Stock Solution: Tris base (30.3 g), glycine (144.0 g), SDS (10 g). Prior to use in electrophoresis, a 1:10 dilution of this stock solution is performed.
8. Standard Marker: BioRad SDS-PAGE standard control 91621 is used as a low M.W. range marker. Contains the following:

Standard	*M.W.* /Da
Phosphorylase	97, 000
Serum albumin	66, 200
*Ovalbumin	45, 000
*Carbonic anhydrase	31, 000
Trypsin inhibitor	21, 000
*Lysozyme	14, 400

* glycosylated – gives positive result on Con A-Blot

2.2. Protein Staining/Destaining

1. Bio-Safe G250 Coomassie Brilliant Blue stain (Bio-Rad, USA).
2. Rapid Silver Stain based on the Amersham Biosciences Plus One Silver Stain Kit.
3. 1:1 (v/v) solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate.

2.3. Lectin Blotting

1. 10x Transfer buffer stock solution: 1.32 M glycine (144.1 g/L), 250 mM Tris (30.3 g/L). The stock solution is diluted 1:10 prior to use with 0.5:3.5:1 ratio, stock solution:DDI H_2O:methanol.
2. Concanavalin A Lectin Blot: Concanavalin A is stored at −20°C (1 mg/mL).
3. Tris-buffered saline with Tween (TBST) buffer: 20 mM Tris (2.4 g/L), 150 mM NaCl (8.7 g/L), adjust to pH 7.5 with 25% HCl, 0.1 mM $MnCl_2$ (12.6 mg/L), 0.1 mM $CaCl_2$ (11.1 mg/L), 0.1% Tween 20 (1 mL/L).
4. Developing solution: 7 mL 4-chloro-1-naphthol (3 mg/mL in methanol, prepare freshly), 39 mL DDI H_2O, 2 mL 0.2 M Tris-HCl, pH 7.5, 20 μL 30% H_2O_2 (add directly before use).

2.4. In-Gel Reductive β-Elimination

1. β-elimination reagent: a freshly made aqueous solution of 0.1 M $NaBH_4$ in 0.3 M NaOH.
2. Glacial acetic acid.
3. Heating block.
4. Vacuum centrifuge.

2.5. In-Gel Nonreductive β-Elimination in Aqueous Ammonia

1. β-elimination reagent: 25% aqueous ammonium hydroxide solution.
2. Heating block.
3. Vacuum centrifuge.

2.6. Acid-Catalyzed Acetylation

1. Round-bottomed glass tubes (11 × 100 mm) with Teflon™-lined screw top.
2. Acetylation reagent: a freshly made mixture of trifluoroacetic acid anhydride (2 vols), glacial acetic acid (1 vol) mixed in a round glass bottomed tube, capped and allowed to cool to room temperature.
3. Vacuum centrifuge.
4. Dichloromethane.

2.7. De-O-Acetylation

1. 1:1 (v:v) conc. NH_4OH/methanol.
2. Heating block.

2.8. Per-O-Methylation

1. 99.9 % A.C.S. dimethyl sulfoxide.
2. NaOH pellets.
3. Pestle and mortar.
4. Iodomethane – as a methylating reagent, this has potential to be a carcinogen, and so should be used with caution; protective clothing should be worn and, since it is volatile, it should be used in a fume cupboard.
5. Freshly made sodium thiosulfate solution, 100 mg/mL in water.
6. Dichloromethane.

3. Methods

3.1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The instructions that follow are based on separating bovine submaxillary gland mucins Type I-S (BSM) (Sigma, USA) using NuPAGE™ 10% Bis-Tris precast gels (1.0 mm × 10 well) (Invitrogen, USA) in an Xcell *Surelock*™ Mini-Cell. Other formats are equally appropriate.

1. Insert a precast minigel into the Xcell *Surelock*™ Mini-Cell and remove the pre-inserted comb.
2. Dissolve the standard (glyco)protein mixture in NuPAGE® LDS sample buffer (4x) and NuPAGE® reducing agent (10x) with quantities between 5 and 25 µg of protein.
3. Heat the mixture at 70°C for 10 min.
4. Prepare SDS running buffer by adding 50 mL NuPAGE® 2-morpholinethane-sulfonic acid (MES) to 950 mL of HPLC grade water. Fill the lower buffer chamber of the Invitrogen Xcell *Surelock*™ Mini-Cell.
5. Add 500 µL NuPAGE® antioxidant solution to 200 mL 1x SDS running buffer and fill the upper buffer chamber of the Xcell *Surelock*™ Mini-Cell.
6. Load 20 µL of each sample into separate wells (*see* **Note 1**). Include in one well a prestained molecular weight marker.

7. Complete the assembly of the gel unit and connect to a power supply set at 200 V for 60 min.
8. The gel unit is disconnected from the power supply and disassembled. The stacking gel is removed and discarded. Cut a small triangle about 0.5 cm in from the top right corner of the resolving gel to allow the gel's orientation to be tracked.

3.2. Protein Staining/Destaining (*see* **Note 2**)

(Glyco)proteins can be visualised by staining with Bio-Safe G250 Coomassie Brilliant Blue (Bio-Rad, USA) or by silver staining.

3.2.1. Staining with Coomassie Brilliant Blue

1. In the case of Coomassie Brilliant Blue staining, simply add enough of the commercially preprepared stain to submerge the gel, which is in an appropriate-sized tray.
2. Place the tray on a rocking platform for 30 min.
3. Pour off the stain.
4. Wash the gel with water for 30 min using a rocking platform. Remove the water and repeat the wash step.
5. After visualisation, excise individual Coomassie-stained protein bands from the gel by manually cutting out the appropriate bands, and place into separate 1500 µL microcentrifuge tubes.
6. Dehydrate the gel piece in the microcentrifuge tube using a vacuum centrifuge.

3.2.2. Silver Staining

To visualise the proteins with silver staining, mass spectrometry-compatible Rapid Silver Stain based on the Amersham Biosciences PlusOne Silver Stain Kit is an appropriate stain to use. (*see* **Note 3**)

1. Prepare the fixing solution by mixing 100 mL ethanol with 25 mL concentrated acetic acid and make up to 250 mL with HPLC grade water. Add to the gel and leave for 30 min at room temperature on a rocking platform.
2. Prepare the sensitizing reagent by mixing 75 mL ethanol, 10 mL sodium thiosulfate (5% w/v) and 17 g sodium acetate and make up to 250 mL with HPLC grade water. Add to the gel and leave for 30 min at room temperature on a rocking platform.
3. Pour off the sensitizing reagent solution.
4. Wash the gel for 5 min with HPLC-grade water. Repeat twice.
5. Prepare the silver reagent by mixing 25 mL silver nitrate (2.5% w/v) with HPLC-grade water to give a final volume of 250 mL and add to the gel for 20 min.
6. Pour off.
7. Wash the gel with water for 30 min using a rocking platform. Remove the water and repeat the wash step.
8. Prepare a developing solution by mixing 6.25 g sodium carbonate with 0.05 mL formaldehyde (37% w/v) and make up to 250 mL with HPLC-grade water. Add to the gel for 5–15 min while on a rocking platform.

9. Prepare a stopping solution by dissolving 3.65 g EDTA-$Na_2.2H_2O$ in 250 mL with HPLC-grade water. Add to the gel for 10 min.
10. After visualisation, excise individual silver stained protein bands from the gel by manually cutting out the appropriate bands and place into separate 1500 µL microcentrifuge tubes.
11. Destain using 1:1 (v/v) solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate by adding 50 µL of this solution to each of the 1500 µL microcentrifuge tubes that contain the excised protein gel bands. Perform occasional vortexing. The colour of the gel bands is monitored until the brownish colour disappears.
12. The gel pieces are subsequently washed several times with HPLC-grade water.
13. Add 100 µL 200 mM ammonium bicarbonate solution to the gel pieces for 20 min and then discard.
14. Wash the gel pieces with HPLC-grade water and then dehydrate with changes of acetonitrile (Fischer Scientific, Loughborough) until the gel pieces turn opaque white.

3.3. Lectin Blotting

To identify potential glycoprotein bands, prepare a second gel of the same samples. This second SDS-PAGE-separated sample is then transferred by electroblotting the separated proteins on to a polyvinylidene fluoride membrane support activated with methanol, and incubated with Concanavalin A lectin. These directions are based on the use of an Invitrogen Xcell II™ blot module to perform the western blot, which is placed in the Invitrogen Xcell *SureLock*™ Mini-Cell buffer chamber.

1. Prepare a 10x transfer buffer stock solution (1.32 M glycine, 250 mM Tris) and dilute 1:10 with 0.5:3.5:1 ratio stock solution:DDI H_2O:methanol prior to use.
2. Prepare a tray of transfer buffer large enough to lay out a transfer cassette with its pieces of foam and with two sheets of 3 MM paper submerged on one side. Cut a sheet of PVDF membrane so that it is larger than the size of the separating gel and lay it on the surface of a separate tray of distilled water and allow the membrane to wet via capillary action. Then submerge the membrane in the transfer buffer on top of the 3 MM paper. Lay the resolving gel on top of the PVDF membrane.
3. Wet two further sheets of 3 MM paper in the transfer buffer and carefully lay them on top of the gel, ensuring that no bubbles are trapped in the resulting sandwich. Lay the second wet foam on top and close up the cassette.
4. Insert the cassette into the buffer chamber ensuring that the PVDF membrane is located between the gel and the anode.
5. Assemble the apparatus prior to connecting to the power supply. Transfer can be achieved using 30 V constant for 1 h using a Bio-Rad Power Pac 1000.
6. Once the transfer is complete, remove the cassette from the buffer chamber and disassemble it.

7. Prior to incubating the blot with Concanavalin A lectin, wash the PVDF membrane 3 times with TBST buffer.
8. Incubate the membrane with 100 µg lectin/20 mL TBST buffer at room temperature with constant shaking for 1 h.
9. Wash the membrane 5 times with DDI H_2O.
10. Prepare the developing solution and develop the blot by incubating the membrane for 15 min in the dark.
11. Wash the blot in H_2O for 1 min and then dry the blot between two pieces of Whatman No. 1 paper.

3.4. In-Gel Release Using Reductive β-Elimination of O-Glycans

1. Place the excised dehydrated gel piece (*see* **Note 4**) into a 1500 µL microcentrifuge tube and add 70 µL 0.5 M $NaBH_4$ in 0.3 M NaOH Incubate at 50°C for 16 h (*see* **Note 5**). Quench the reaction by adding glacial acetic acid dropwise, until the evolution of hydrogen gas ceases.
2. Remove the liquid surrounding the gel piece (*see* **Note 6**) and place in a round-bottomed glass tube with a Teflon™-lined screw cap.
3. Wash the gel piece several times with 100 µL HPLC grade water with a few seconds of vortexing.
4. Pool the water washes with the original liquid from around the gel piece. Dry the combined extracts using a vacuum centrifuge.

3.5. β-Elimination in Aqueous Ammonia (*see* Note 7)

Place the excised dehydrated gel piece into a 1500 µL microcentrifuge tube and add 70 µL 25% NH_4OH and incubate at 45°C for 16 h.

3.6. Acetylation of the Dried Extracted Material (*see* Notes 8 and 9)

1. Acetylate the dried extracted material by adding 250–500 µL acetylation reagent to the dry sample, cap tube and mix well.
2. Allow the reaction to proceed at room temperature for 15–20 min.
3. Evaporate to dryness in a vacuum centrifuge or under a stream of nitrogen.
4. Use 1 mL of dichloromethane to redissolve the residue, cap the tube and vortex.
5. Add ~1 mL deionised water, vortex and centrifuge at low speed to separate the phases.
6. Remove the upper aqueous phase with a Pasteur pipette and discard.
7. Repeat **steps 5** and **6**.
8. Evaporate the dichloromethane using a vacuum centrifuge or stream of air or nitrogen.

3.7. De-O-Acetylation (*see* Note 9)

1. Mix dry acetylated material in a round-bottomed glass tube with a Teflon™-lined screw cap with 250–500 µL freshly made 1:1 (v:v) conc. NH_4OH/methanol and incubate at 20–22°C for 18 h.
2. Dry the reaction mixture under nitrogen.

3.8. Per-O-Methylation (*see* Note 9)

1. Dissolve the dry de-*O*-acetylated sample in 0.5 mL 99.9 % A.C.S dimethyl sulfoxide (Sigma-Aldrich, St. Louis. USA) in a round-bottomed, Teflon™-screw-capped glass tube.
2. Rapidly grind a few pellets of NaOH in a pestle and mortar and add two micro-spatulas (~50 mg) of the ground NaOH to the glass tube and a few drops (50–100 µL) of iodomethane using a Pasteur pipette, and recap the tube.
3. Allow to stand for 10 min at room temperature, after which add a further 5–10 drops of iodomethane to the tube.
4. Allow to stand for a further 10 min at room temperature, after which add 20 more drops of iodomethane and recap the tube.
5. After 20 min, quench the reaction by cautiously adding 1 mL freshly made 100 mg/mL sodium thiosulfate solution to the sample.
6. Immediately add 1 mL dichloromethane. Recap the tube and mix the reagents thoroughly to form an emulsion.
7. Centrifuge the sample for 10–15 s. Remove the upper aqueous layer with a pipette and then wash the dichloromethane three times with water. During each wash, form an emulsion, followed by centrifuging for 10–15 s to separate the phases and then remove the upper aqueous phase.
8. After the third washing, dry the dichloromethane under nitrogen.

3.9. Mass Spectrometry of Per-O-Methylated and Per-O-Acetylated Released O-Glycans

The following conditions are based on acquiring mass spectra on an Applied Biosystems/MDS SCIEX API QSTAR Pulsar i (Ontario, Canada) quadrupole orthogonal acceleration time of flight tandem mass spectrometer. Other mass spectrometers are equally appropriate to use.

3.9.1. Electrospray

1. For electrospray applications the ion source contains a microelectrospray arm mounted in an Applied Biosystems ion source housing.
2. A 100 µL Hamilton 1710N syringe (1.46 mm inside diameter) was used with the integral syringe driver to deliver the sample, dissolved in methanol (Fischer Scientific, Loughborough), to the ion source in a continuous flow at 1 µL/min.
3. The ion source gas reading is set to 4, the curtain gas to 20 and the capillary is held at 5500 V.
4. The instrument is operated in the positive mode with declustering and focusing potentials of 65 and 265 V respectively.
5. For Pulsar operation the Ion Release Delay is set to 6 and the Ion Release Width to 5.
6. In MS mode, the collision gas is set to read 3 and increased to 6 during tandem MS.
7. The collision energy offset for tandem MS is varied between 25 V and 70 V. Nitrogen is used for both the collision gas and for the curtain gas.

8. The detection of ions is performed using a microchannel plate detector system with a time to digital converter.
9. The data are recorded and analysed using the Applied Biosystems/ MDS SCIEX Analyst QS Software.

The electrospray mass spectrum **(Fig. 1A)** of per-*O*-acetylated glycans released using reductive β-elimination from a bovine submaxiliary mucin SDS-PAGE band, following staining with Coomassie Brilliant Blue, contains an intense $(M+Na)^+$ ion at *m/z* 456 for per-*O*-acetylated HexNAc-ol. A less intense ion at *m/z* 743 is also observed for $(M+Na)^+$ of HexNAc-HexNAc-ol.

The product ion spectrum of the ion at *m/z* 456 **(Fig. 1B)** contains intense fragment ions at *m/z* 314 $(M+H^+ - 2 \times 60)$, 336 $(M+Na^+ - 2 \times 60)$ and 396 $(M+Na^+ - 60)$ and less intense ions at *m/z* 374 $(M + H^+ - 60)$ and 254 $(M+H^+ - 3 \times 60)$, the 60 mass unit loss corresponding to the elimination of the elements of acetic acid. Other confirmatory fragment ions (*m/z* 110 $(M+H^+ - (2 \times 42) - (4 \times 60))$, 152 $(M+H^+ - 42 - (4 \times 60))$, 212 $(M+H^+ - 42 - (3 \times 60))$, 230 $(M+H^+ - (2 \times 42) - (2 \times 60))$, 254, 272 $(M+H^+ - 42 - (2 \times 60))$, 314, 336, 374 and 396) are also present.

Tandem mass spectrometric analysis of the precursor ion at *m/z* 743 **(Fig. 1C)** contains intense fragment ions at *m/z* 683 $(M + Na^+ - 60)$, 623 $(M+Na^+ - 2 \times 60)$ and 563 $(M+Na^+ - 3 \times 60)$, and two less intense fragment ions at *m/z* 601 $(M+H + - 2 \times 60)$ and 541 $(M+H^+ - 3 \times 60)$. There are less intense fragment ions at *m/z* 352, 370 and 414, which correspond to diagnostic sodiated carbohydrate fragment B_1, C_1 and Y_1 ions respectively for a per-*O*-acetylated HexNAc-HexNAc-ol.

Mass spectrometric analysis of the glycans released from a silver-stained BSM glycoprotein gel band also resulted in the detection of ions at *m/z* 456 and 743 corresponding to the HexNAc-ol and HexNAc-HexNAc-ol BSM glycans. Product ion analyses of these ions yielded fragment ions indistinguishable from those produced on CID of the $(M+Na)^+$ per-*O*-acetylated HexNAc-ol and HexNAc-HexNAc-ol species released from the Coomassie stained gel (**Fig. 1B** and **1C**).

3.9.2. MALDI

1. When the QSTAR is being operated with the MALDI source a standard stainless steel PerSeptive flat microtiter target plate sample introduction system is used.
2. The dried sample is dissolved in methanol (typically 5–10 μL).
3. The dried-droplet method is used for preparing the sample-matrix spots. 1 μL sample solution is mixed with 1 μL of 10 mg mL^{-1} α-cyano-4-hydroxycinnamic acid (in 50 % aqueous acetonitrile 0.1 % TFA solution) in a plastic, 1500 μL microcentrifuge tube, in order to produce a matrix to sample ratio of about 5000:1. An aliquot of this mixture (0.5–2.0 μL) is then applied to the stainless steel sample target and allowed to air dry.

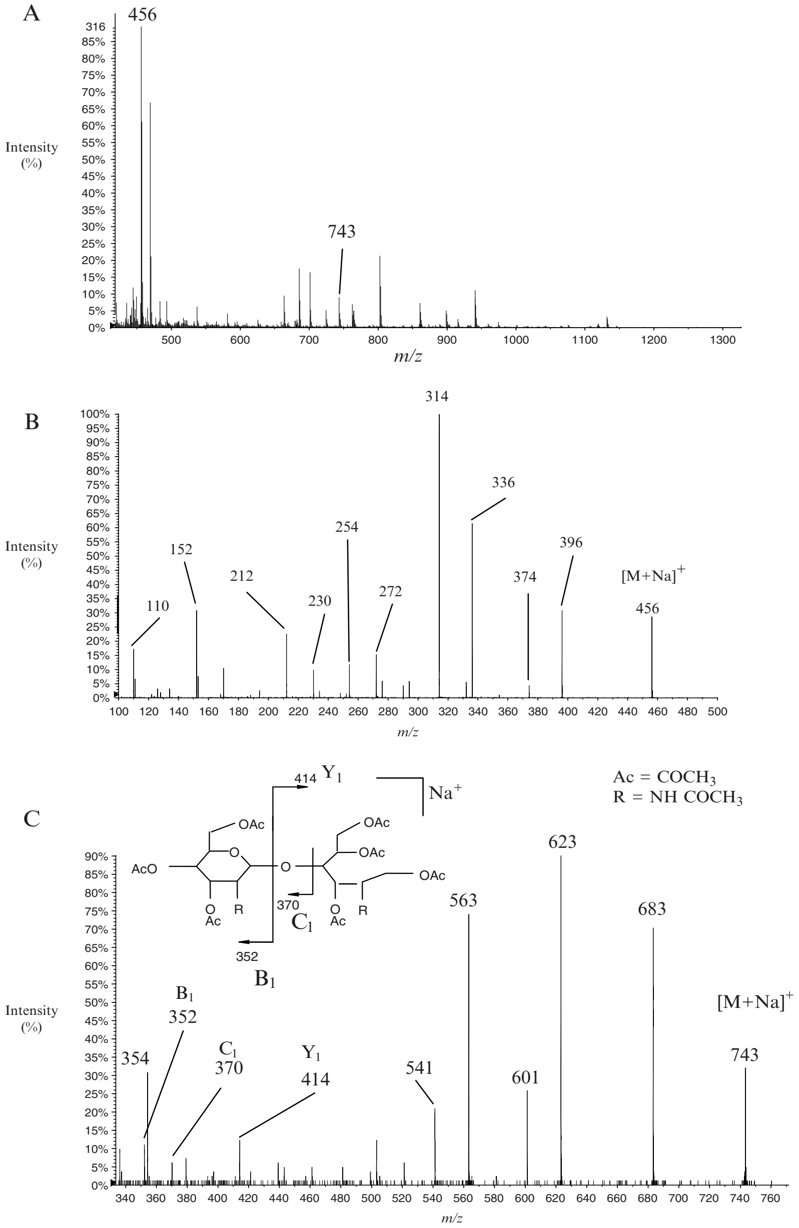

Fig. 1. Electrospray Mass Spectra of Per-*O*-Acetylated Glycans Released In-Gel using Reductive β-Elimination from an SDS-PAGE-separated Bovine Submaxillary Mucin Glycoprotein. **(A)** Positive ion mode electrospray mass spectrum recorded in methanol; **(B)** CID product ion mass spectrum of $(M+Na)^+$ Per-*O*-Acetylated HexNAc-ol ion at *m/z* 456; **(C)** CID product ion mass spectrum of $(M+Na)^+$ Per-*O*-Acetylated HexNAc-HexNAc-ol ion at *m/z* 743 (Reproduced with permission from ***ref. 11***).

4. Ionisation of the matrix and sample is performed using a nitrogen UV laser with a wavelength of 337 nm, 1 to 30 Hz pulsing rate and 200 μJ laser power output, although for most applications a laser power of 25 μJ is sufficient.
5. The data are recorded and analysed using the Applied Biosystems/MDS SCIEX Analyst QS Software. The laser power and plate position are controlled using the Applied Biosystems oMALDI Server 2.2 software. Instrumental and detection parameters used are similar to those described above for electrospray operation.

4. Notes

1. The smallest amount of BSM protein that could be applied to a gel yielding a band from which a BSM glycan could be detected by ESI-MS after in-gel reductive β-elimination and extraction was 5 μg **(Fig. 2)**.
2. Not performing destaining of the Coomassie stained bands does not seem to have any adverse effect on the chemical de-*O*-glycosylation and detection of the released glycans. On the other hand, destaining of silver stained

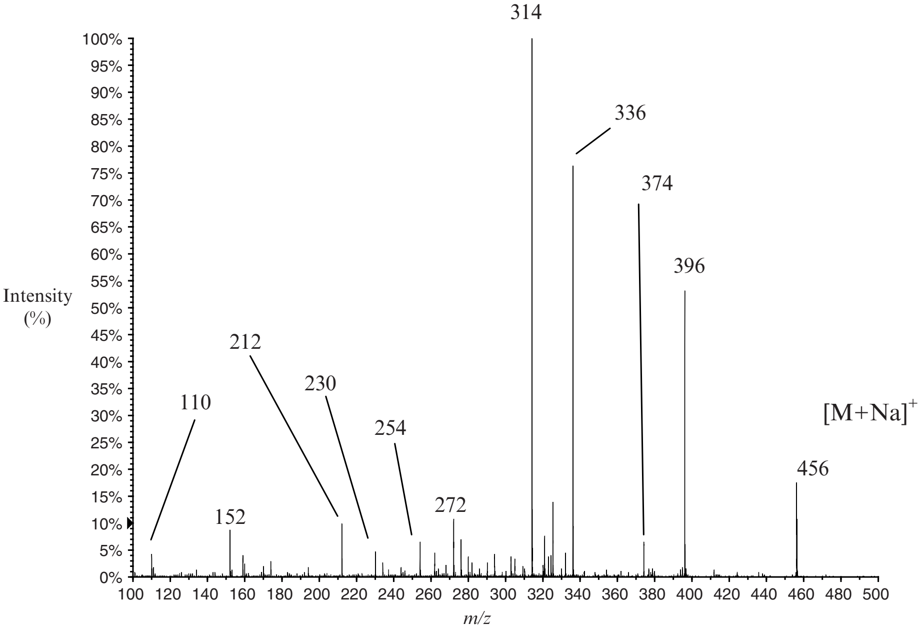

Fig. 2. Electrospray CID Product Ion Mass Spectrum of HexNAc-ol from an SDS-PAGE BSM Glycoprotein Band, visualised using silver staining after In-Gel De-*O*-Glycosylation and Per-*O*-Acetylation. The BSM glycoprotein band was excised from a gel lane that had 5 μg BSM applied to it. (Reproduced with permission from ***ref. 11***).

bands is required prior to chemical de-*O*-glycosylation. The removal of SDS prior to chemical de-*O*-glycosylation does not appear to have an effect on the chemical release or detection of *O*-linked glycans.

3. Silver staining provides more sensitive staining of proteins than does Coomassie Brilliant Blue and is a very widely used staining technique. In silver stained gels, glycan release appears not to be as efficient as that obtained from a BSM glycoprotein in a band that has been visualised by Coomassie staining, since poorer signal:noise ratios for the $(M+Na)^+$ per-*O*-acetylated HexNAc-ol and HexNAc-HexNAc-ol ions (*m/z* 456 and 743) were obtained. Silver staining relies on cross-linkage in the gel, which may restrict access of the reagent to the protein, or restrict the extraction of released glycans from the gel, as well as potentially modifying the glycans. We therefore propose that this may explain the poorer signal:noise of the ions observed on in-gel release using silver stained bands.
4. The hydration state of the gel piece prior to the addition of the β-elimination reagent has the biggest impact of all experimental variables on the effectiveness of glycan release. It is preferable to have the gel piece dried (we use a vacuum centrifuge) prior to performing the in-gel de-*O*-glycosylation. This is understandable, as in this way there is no dilution of the reagent. In addition, if the gel piece is dried, the aqueous reagent readily enters the gel, so accessing the protein more easily. We have noted that the use of dried bands provides an increase in the glycan release over shorter reaction times.
5. The strength of the alkali and the reaction time have less of an impact on the extent of glycan release; the intensity of glycan ions is not increased by increasing reaction time and reagent concentration. Indeed, longer reaction times may allow the alkaline conditions to destroy the glycan, or may produce something that interferes with either the extraction of the glycans or their mass spectrometric detection. A shorter reaction time provides for better detection of the glycan ions. A compromise between losses of disaccharide and failure to release it was achieved by carrying out glycan release reactions for no longer than 16 h.
6. Released glycans were effectively extracted from the gel pieces by vortexing in water; no improvement in the amount of glycan extracted with use of further extractions with 1:1 v:v acetonitrile/water was achieved
7. In-gel nonreductive β-elimination release has been performed on a gel band from a Coomassie Brilliant Blue stained SDS-PAGE separation of *M. avium* capsular proteins. This was then followed by in-gel tryptic digestion of the residual polypeptide following glycan release. Analysis of the

tryptic peptides using a RP capillary monolithic column coupled off-line with MALDI-ToF/ToF analysis, yielded an NCBI database hit for an *M. avium* protein, by matching two peptides. The significance of this modified approach is that using ammonium hydroxide to release the glycans nonreductively from the protein in-gel is compatible with subsequent in-gel tryptic digestion of the protein, enabling identification of the protein from its released peptides. It is also a convenient means of labelling the amino acid in the protein to which the glycan had been attached, thus having the potential to allow glycosylation site analysis ***(9)***. This variant of our approach could therefore be used not only to profile the glycan moieties but also to identify the protein and determine the site of glycan attachment as part of a gel-based proteomics experiment, allowing comprehensive glycoprotein characterisation.

8. The extraction into dichloromethane acts as a sample clean-up step to remove water-soluble reagents and interferents ***(13)***.
9. While per-*O*-acetylation is readily carried out in the presence of a range of salts and other reagents ***(13)***, the mass spectrometric response of peracetylated glycan is not as good as that of the permethylated derivative, and the mass spectrometric fragmentation tends to be less informative. However, the permethylation reaction is less tolerant of noncarbohydrate impurities, and so is best carried out on a purer sample – peracetylation and organic-aqueous partition is a convenient way to produce an appropriately purified sample. Although permethylation is carried out in strongly basic conditions, we have observed that better data were obtained if the peracetylated glycan was first de-*O*-acetylated prior to permethylation.
10. To demonstrate the utility of the developed in-gel de-*O*-glycosylation procedure, the reductive β-elimination method has been applied to a protein gel band (**Fig. 3A**, band X) from a Coomassie-stained SDS-PAGE separation of capsular glycoproteins from *M. avium* strain 2151 with smooth transparent (SmT) morphotype ***(11)***. The band chosen had produced a positive result in a duplicate Concanavalin A lectin blot, which suggested that this ~32 kDa protein was glycosylated. The MALDI mass spectrum of the glycans obtained after per-*O*-acetylation did not contain any ions for per-*O*-acetylated glycans. The mixture was de-*O*-acetylated and per-*O*-methylated in order to improve the mass spectrometric response, and analysed using ESI-MS. An ion at *m/z* 493 for $(M+Na)^+$ of Hex_1Hex-ol was observed. Tandem mass spectrometric analysis of the ion at *m/z* 493 **(Fig. 3B)** yielded an intense ion at *m/z* 275 for the diagnostic sodiated Y_1 carbohydrate fragment ion for Hex-Hexitol.

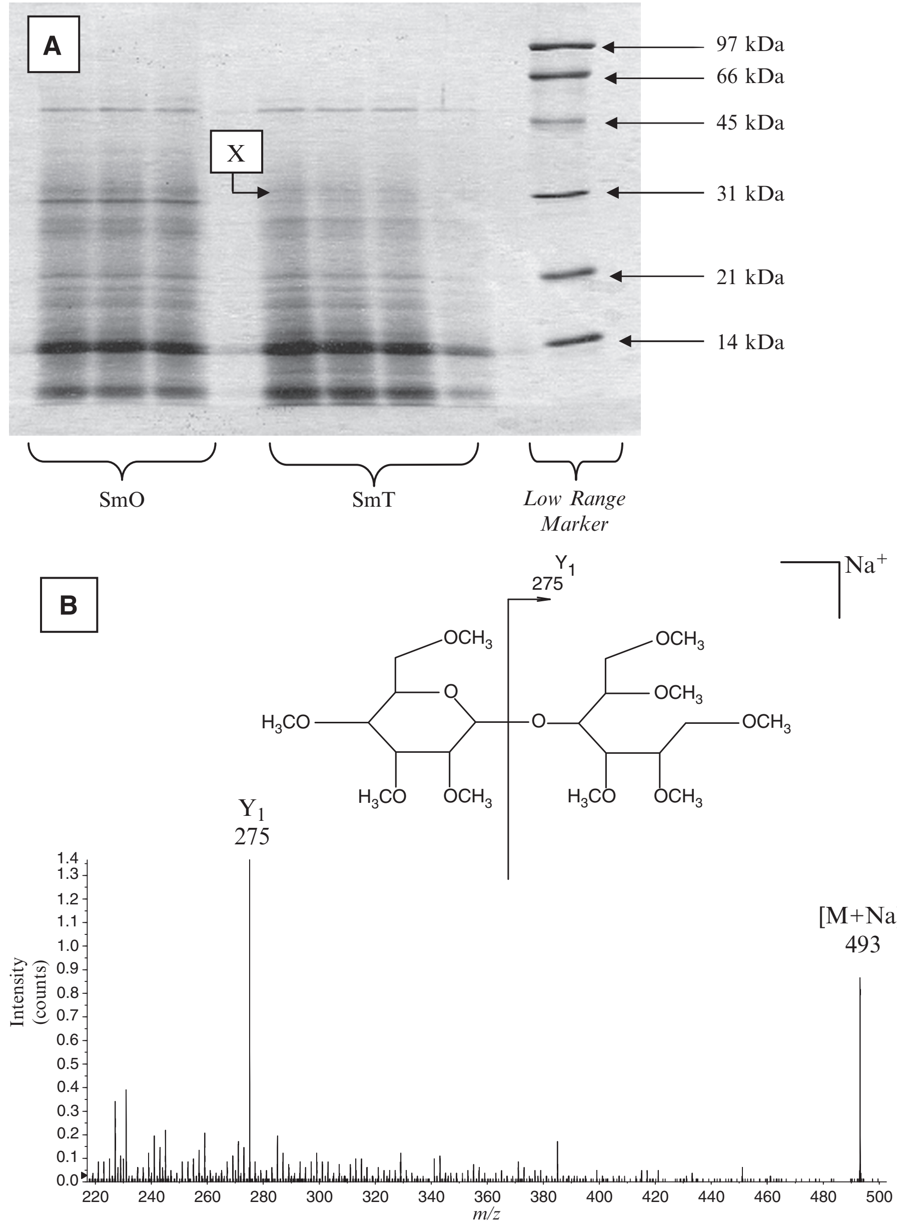

Fig. 3. **(A)** Coomassie Stained SDS-PAGE Separation of Proteins Fractionated from the Capsules of Two *M. avium* Morphotypes, Smooth Transparent (SmT) and Smooth Opaque (SmO). **(B)** Positive ion mode CID tandem electrospray mass spectrum of $(M+Na)^+$ per-*O*-methylated Hex_1Hexitol ion at *m/z* 493 obtained from *M. avium* 2151 SmT Capsular Glycoprotein After In-Gel De-*O*-Glycosylation and Per-*O*-Methylation. (Reproduced with permission from ***ref. 11***).

Acknowledgments

We are very grateful to Prof Otto Holst and Astrid Gruhl of the Research Center Borstel, Borstel, Germany, for the productive and very enjoyable *Mycobacterium avium* collaboration, and to Astrid for carrying out the SDS-PAGE shown in Figure 3A and providing band X. We gratefully acknowledge AMT's studentship provided by the Royal Society of Chemistry (RSC) and the Engineering and Physical Sciences Research Council (EPSRC), and the British Council (ARC project 1126) and the Deutscher Akademischer Austauschdienst (DAAD) (ARC project ARC-Holst) for funding the Anglo-German collaboration. JTO gratefully acknowledges funding from the Analytical Chemistry Trust Fund, the RSC Analytical Division and EPSRC.

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Glycopeptide Analysis Using LC/MS and LC/MS^n

Site-Specific Glycosylation Analysis of a Glycoprotein

Satsuki Itoh, Daisuke Takakura, Nana Kawasaki, and Teruhide Yamaguchi

1. Introduction

A glycoprotein consists of heterogeneous molecules attached to diverse oligosaccharides at multiple glycosylation sites. Glycosylation analysis at each glycosylation site is important for understanding the function of carbohydrate moiety in glycoproteins. MS of glycopeptides is a commonly used method for the analyses of glycosylation sites and site-specific glycosylations ***(1–4)***. Since glycopeptide ions sometimes fail to be acquired by MS in the presence of excess peptides due to their lower ionization efficiency, separation or enrichment of glycopeptides is crucial for the MS of glycopeptides to be performed. LC/MS that allows for separation of glycopeptides from a mixture of peptides and acquisition of their mass spectra is one of the most effective means in use for the site-specific glycosylation analysis of glycoproteins.

Fig. 1 outlines the site-specific glycosylation analysis by using LC/MS and LC/MS^n. Disulfide bonds in the glycoproteins are reduced and alkylated with monoiodoacetic acid to facilitate the complete digestion with proteinases (*see* **Subheading 3.1.1**). The alkylated glycoprotein is incubated with an appropriate proteinase that provides glycopeptides containing a single glycosylation site (*see* **Subheading 3.1.2**). The complex mixture of peptides is separated by reversed-phase LC and subjected to on-line electrospray ionization (ESI) MS (*see* **Subheading 3.2.1**). The predominant ions acquired are automatically subjected to MS^n for sequencing of carbohydrates and peptides (*see* **Subheading 3.2.2**). After acquisition of the data, the MS/MS spectra of glycopeptides are picked out by matching the all acquired MS/MS spectra for the predicted MS/MS

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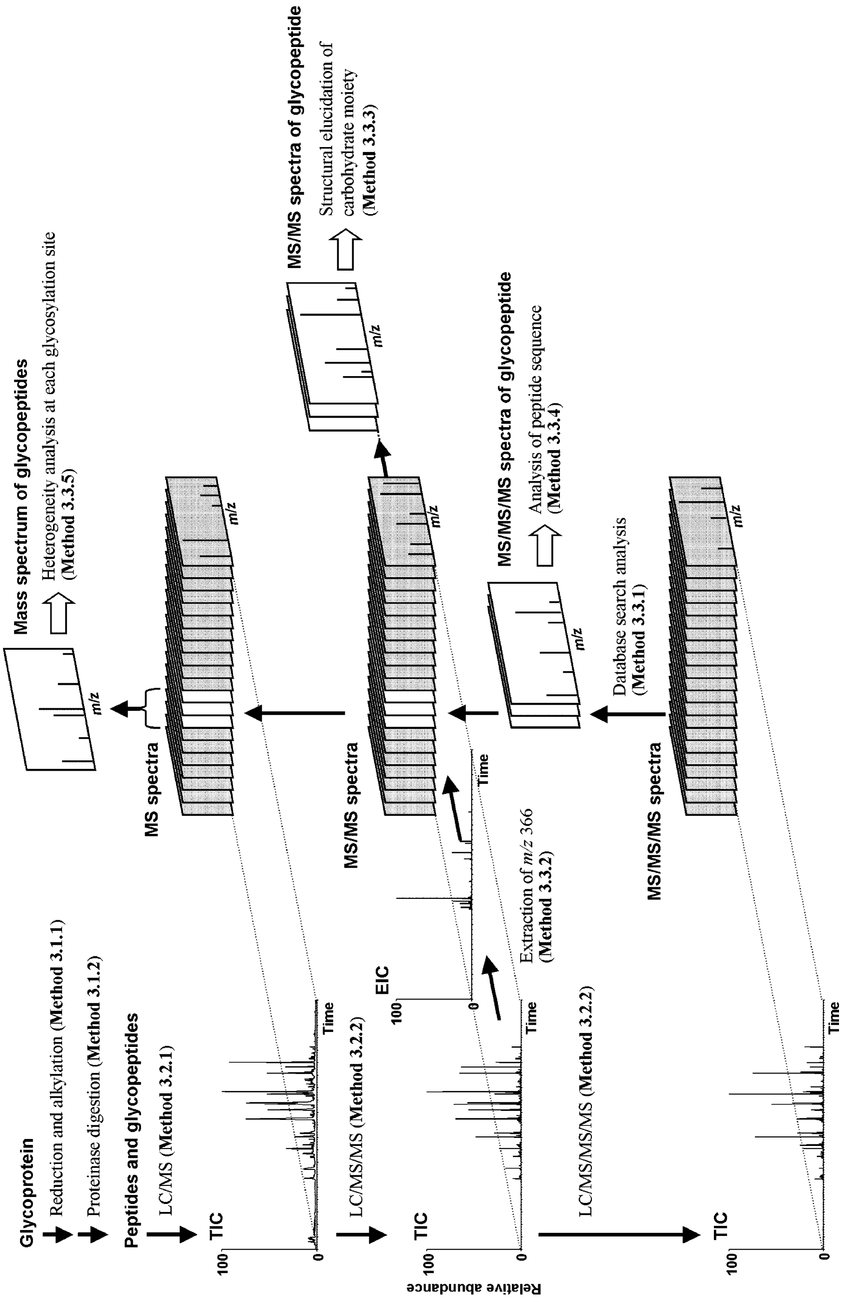
Glycoprotein
Reduction and alkylation (Method 3.1.1)
Proteinase digestion (Method 3.1.2)
Peptides and glycopeptides
LC/MS (Method 3.2.1)
TIC
100
0
Time
Relative abundance
MS spectra
m/z
Mass spectrum of glycopeptides
Heterogeneity analysis at each glycosylation site
(Method 3.3.5)
LC/MS/MS (Method 3.2.2)
TIC
100
0
Time
EIC
100
0
Extraction of m/z 366
(Method 3.3.2)
Time
MS/MS spectra
m/z
MS/MS spectra of glycopeptide
Structural elucidation of carbohydrate moiety
(Method 3.3.3)
m/z
Database search analysis
(Method 3.3.1)
MS/MS/MS spectra of glycopeptide
Analysis of peptide sequence
(Method 3.3.4)
LC/MS/MS/MS (Method 3.2.2)
TIC
100
0
Time
MS/MS/MS spectra
m/z

Fig. 1. Flow chart of typical site-specific glycosylation analysis by LC/MS and LC/MS^n.

spectra of the object glycoprotein by using a search engine (*see* **Subheding 3.3.1**) *(5)*. Even if database search analysis failed to locate the MS/MS spectra of glycopeptides, tracing carbohydrate-distinctive ions, such as HexNAc$^+$ (*m/z* 204) and Hex-HexNAc$^+$ (*m/z* 366), acquired in MS/MS provides a clue to locate the desired spectra (*see* **Subheding 3.3.2**) *(6,7)*. The carbohydrate structure can be deduced from the fragment pattern appearing in the extracted MS/MS spectra (*see* **Subheding 3.3.3**). The peptide-related ions, which bear reducing-end HexNAc at peptides ((Peptide-HexNAc + H)$^+$), often arise from the glycopeptide molecular protonated ions. The peptide sequence can be estimated by further MS^n acquired from the peptide-related ions as precursors (*see* **Subheding 3.3.4**). Since glycopeptides containing identical sequences are retained at close position, preliminary site-specific carbohydrate heterogeneity can be estimated from the integrated mass spectrum acquired at the glycopeptide peaks (*see* **Subheding 3.3.5**).

Tissue-plasminogen activator (t-PA) is a secreted serine protease which converts the plasminogen to plasmin, a fibrinolytic enzyme. This protease consists of 527 amino acid residues and is supposed to be digested into 51 peptides by trypsin **(Fig. 2)**. Previous studies suggest the fucosylation at Thr61 (peptide T8), the attachment of high-mannose type oligosaccharides to Ans117 (peptide T11),

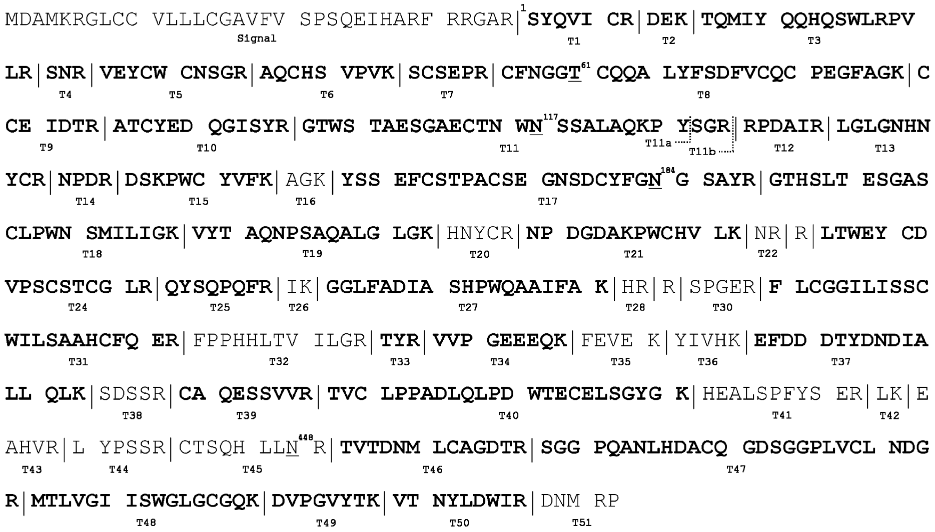
MDAMKRGLCC VLLLCGAVFV SPSQEIHARF RRGAR|¹SYQVI CR|DEK|TQMIY QQHQSWLRPV
Signal
T1
T2
T3
LR|SNR|VEYCW CNSGR|AQCHS VPVK|SCSEPR|CFNGGT⁶¹ CQQA LYFSDFVCQC PEGFAGK|C
T4
T5
T6
T7
T8
CE IDTR|ATCYED QGISYR|GTWS TAESGAECTN WN¹¹⁷SSALAQKP YSGR|RPDAIR|LGLGNHN
T9
T10
T11
T11a
T11b
T12
T13
YCR|NPDR|DSKPWC YVFK|AGK|YSS EFCSTPACSE GNSDCYFGN¹⁸⁴G SAYR|GTHSLT ESGAS
T14
T15
T16
T17
CLPWN SMILIGK|VYT AQNPSAQALG LGK|HNYCR|NP DGDAKPWCHV LK|NR|R|LTWEY CD
T18
T19
T20
T21
T22
VPSCSTCG LR|QYSQPQFR|IK|GGLFADIA SHPWQAAIFA K|HR|R|SPGER|F LCGGILISSC
T24
T25
T26
T27
T28
T30
WILSAAHCFQ ER|FPPHHLTV ILGR|TYR|VVP GEEEQK|FEVE K|YIVHK|EFDD DTYDNDIA
T31
T32
T33
T34
T35
T36
T37
LL QLK|SDSSR|CA QESSVVR|TVC LPPADLQLPD WTECELSGYG K|HEALSPFYS ER|LK|E
T38
T39
T40
T41
T42
AHVR|L YPSSR|CTSQH LLN⁴⁴⁸R|TVTDNM LCAGDTR|SGG PQANLHDACQ GDSGGPLVCL NDG
T43
T44
T45
T46
T47
R|MTLVGI ISWGLGCGQK|DVPGVYTK|VT NYLDWIR|DNM RP
T48
T49
T50
T51

Fig. 2. Amino acid sequence of t-PA and tryptic peptides T1-T51. Boldface type indicates the peptides identified by the database search analysis with the search engine: SEQUEST (Thermo Fisher Scientific). Potential *N*-glycosylation sites are underlined. Thr61 is fucosylated.

and the linkage of sialylated complex type oligosaccharides to Asn 184 and 448 (peptide T17 and T45) ***(8)***. Using t-PA as a model glycoprotein, we here illustrate the protocol for the mass spectrometric peptide/glycopeptide mapping for the site-specific glycocylation analysis of a glycoprotein.

2. Materials

2.1. Sample Preparation

2.1.1. Reduction and Carboxymethylation

1. Buffer: 0.5 *M* Tris-HCl (pH8.6) containing 8 *M* guanidine hydrochloride and 5 m*M* EDTA.
2. Reducing reagent: 2-mercaptothanol, dithiothreitol, and Tris(carboxyethyl)phosphin (TCEP) are used instead.
3. Alkylation reagent: sodium iodoacetate (5 mg) is suspended in 40 µl of the reduction and carboxymethylation buffer. Iodoacetamide is used instead.
4. Gel filtration column for desalting: Sephadex G-25 column (e.g., NAP-10, and PD-10 column, GE Healthcare).
5. Freeze-drying device or vacuum centrifuge (e.g., SpeedVac concentrator).

2.1.2. Proteolytic Digestion

1. Proteinase: Trypsin, Lys-C, Glu-C and Asp-N are commonly used. For the tryptic digestion, Trypsin-Gold (Promega, Madison, WI, USA) is dissolved in 50 m*M* acetic acid at the concentration of 1 mg/ml (*see* **Note 1**). The solution is divided into several tubes and stored at −70°C.
2. Tryptic digestion buffer: 0.1 *M* Tris-HCl (pH 8.0).

2.2. LC/MS^n

1. LC equipment: HPLC system capable of binary gradient formation at a flow rate of 0.3–5.0 µl/ml.
2. On-line cartridge: A reversed-phase cartridge guard column (e.g., Microcolumn, C18, 0.3 mm × 5 mm, particle size 5 µm, Chemicals Evaluation and Research Institute, Japan.).
3. Column: A reversed-phase column (0.075–0.3 mm i. d. × 50–150 mm length, 3 or 5 µm particle size). A C18 or C30 column is commonly used for the peptide/glycopeptide mapping (e.g., L-column, 0.075 mm i. d. × 150 mm length, particle size 3 µm, Chemicals Evaluation and Research Institute). A graphitized carbon column (e.g., Thermo Fisher Scientific, Waltham, MA, USA) can be used for the analysis of hydrophilic glycopeptides.
4. Nanospray tip and x-y-z translational stage: Commercially available from analytical instrument manufacturers (e.g., New Objective, Inc., Woburn, MA, USA).
5. Ion trap type mass spectrometer equipped with nanoESI source (e.g., LTQ, Thermo Fisher Scientific).

6. LC mobile phase: HPLC grade H_2O, acetonitrile and formic acid. Solvent A: 0.1% formic acid in 2% acetonitrile. Solvent B: 0.1% formic acid in 90% acetonitrile.

2.3. Data Analysis

1. Search engine: e.g., TurboSEQUEST (Thermo Fisher Scientific) or Mascot (Matrixscience, London, UK).
2. Database: Swiss-Prot or NCBI-nr.

3. Methods

3.1. Sample Preparation

3.1.1. Reduction and Alkylation of a Glycoprotein

1. A glycoprotein (10 µg) is dissolved in 68 µl of reduction and carboxymethylation buffer.
2. To the solution, 2-mercaptethanol (0.5 µl) is added. The mixture is incubated at room temperature for 2 h.
3. Sodium monoiodoacetate solution (11.3 µl) is added to the sample solution, and the mixture is incubated at room temperature for 2 h in the dark.
4. The reaction mixture is applied on a Sephadex G-25 column equilibrated with water. The excess reagent is passed through the filter to remove.
5. Elution was carried out with water, and the eluant containing the alkylated glycoprotein is collected.
6. The eluant is lyophilized.

3.1.2. Proteolytic digestion

1. The alkylated glycoprotein is dissolved in 100 µl of proteolytic digestion buffer.
2. Trypsin solution (0.5 µl) is added to the sample solution. The mixture is incubated at 37°C for 16 h.
3. The mixture is stored at −20°C until just before the analysis.

3.2. LC/MS^n

3.2.1. LC/MS

1. The column is equilibrated by running 98% of solvent A through it at an appropriate flow rate for 20–30 min (*see* **Note 2**).
2. The nanospray tip is attached to the outlet of the column and set in the x-y-z translational stage. The spray tip is aligned with the capillary of the mass spectrometer for accurate spray direction by moving the x-y-z translational stage.
3. The mass spectrometer (ESI voltage, electron multiplier, gas pressure, and capillary temperature, etc.) is set up and the stable spray is checked with elution buffer.
4. A sample (1 µl) is injected onto the column and both the chromatography gradient and mass spectrometer data collection are simultaneously started (*see* **Notes 3** and **4**). The column is typically eluted by a linear gradient from 2% to 45% of

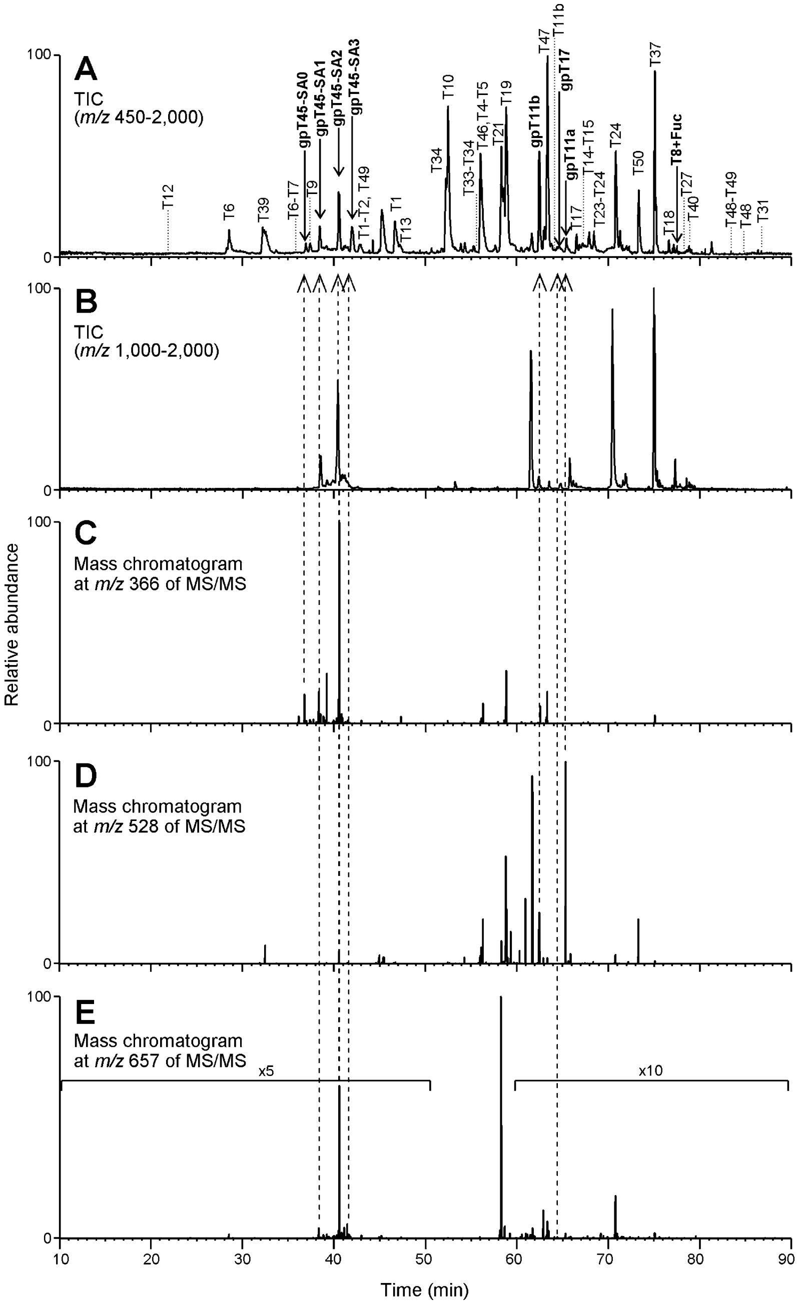

Fig. 3. Mass spectrometric peptide map. (**A**) Total ion chromatogram (TIC) obtained by mass scan (*m/z* 450–2,000) in the positive ion mode. (**B**) TIC obtained by mass scan (*m/z* 1,000–2,000). (**C–E**) Mass chromatograms at *m/z* 366, 528 and 657 obtained by MS/MS

solvent B in 100 min. A mass scan (*m/z* 450–2,000) is performed for the acquisition of both peptides and glycopeptides. Since glycopeptides are relatively large, the mass scan at *m/z* 1,000–2,000 is effective for the specific acquisition of glycopeptides (*see* **Note 5**).

As a typical mass spectrometric peptide map, the total ion chromatogram (TIC) obtained by mass scan of the tryptic digested t-PA at *m/z* 450–2,000 and 1,000–2,000 are shown in **Fig. 3A** and **3B**, respectively.

3.2.2. LC/MS^n

The most intense ion at each mass scan is automatically subjected to MS^n as a precursor ion. Molecular related ions once used as precursor ions are not subjected to MS^n within 30 s.

3.3. Data Analysis

3.3.1. Database Search Analysis for Locating the MS/MS Spectra of Glycopeptides

The MS/MS spectra of glycopeptides are located by matching the acquired MS/MS spectra for the predicted MS/MS spectra from objective glycoprotein in a database using a search engine. *N*-glycosylated peptides are often identified by the database search analysis by using the following parameters: carboxymethylation (58 Da) at Cys, and a possible modification of HexNAc (203 Da) at Asn, and dHex (146 Da) at Ser and Thr *(5)*.

Boldface type in **Fig. 2** indicates the peptides identified by the database search analysis. Nearly 85% of amino acid residues were identified (The peak assignment is illustrated in **Fig. 3A**). Thr61-fucosylated peptide T8 was found around 78 min. Glycosylated and deglycosylated peptide T11b were located around 62 and 64 min. Peptide T17 was identified as the deglycosylation form but not the glycosylated form. The database search analysis did not provide any clues to locate either glycosylated or deglycosylated peptide T45.

◄

Fig. 3. (continued) Sample: Tryptic digest of carboxymethylated recombinant human t-PA (Wako Pure Chemical Industries, Osaka, Japan); Amount of injection, 0.1 µg (1 µl) LC: Column, C18 (L-column, 0.075 mm i. d. × 150 mm length, particle 3 µm, Chemicals Evaluation and Research Institute, Japan); Instrument: Paradigm (Michrom Bioresources, Inc.); cartridge, Micro column (0.3 mm × 5 mm, particle size 5 µm, Chemicals Evaluation and Research Institute); Pump A: 0.1% formic acid/2% acetonitrile; Pump B: 0.1% formic acid/ 90% acetonitrile; flow rate, 300 nl/min; gradient program, 2–45% of B in 100 min. MS: Instrument, LTQ-FT (Thermo Fisher Scientific); Tip: Fortis tip (AMR, Tokyo, Japan); Ion mode, positive.

3.3.2. Location of glycopeptides using carbohydrate-related ions

The MS/MS spectra of glycopeptides can be located by tracing the carbohydrate-related fragment ions acquired in MS/MS. The peaks appearing in mass chromatograms at *m/z* 366 (Hex-HexNAc$^+$) and 657 (NeuAc-Hex-HexNAc$^+$) afford a clue to locate the glycopeptide MS/MS spectra.

Fig. 3C, **3D**, and **3E** show the mass chromatograms of ions at *m/z* 366, 528 and 657, respectively. Glycopeptides T45 and T11a, which were overlooked by the database search analysis, were located by it.

3.3.3. Structural Estimation of Carbohydrate Moiety

The mass and MS/MS spectra are extracted from the acquisition positions of glycopeptides located by **Subheading 3.3.1** and/or **3.3.2**. The sequences of carbohydrates can be deduced from the fragment ions in the MS/MS spectra.

Fig. 4A and **4B** show the mass spectrum of the glycopeptide peak at 62 min and the MS/MS spectrum acquired from one of the protonated ions (*m/z* 1,412) as a precursor, respectively. The carbohydrate-related fragment ions at *m/z* 528, 690, and 852, and the peptide-related ion, (Peptide-HexNAc + 2H)$^{2+}$ (*m/z* 1,611) in **Fig. 4B** suggest the attachment of a high-mannose type oligosaccharide ($Hex_5HexNAc_2$) to peptide T11b (theoretical mass: 3016.33) **(Fig. 4A)**.

3.3.4. Sequencing of Peptide Moiety

The peptide sequence can be confirmed by the b- and y-ions that arise in MS/MS/MS acquired from the peptide-related ions ***(9)***.

Fig. 4C shows the MS/MS/MS spectrum acquired from (Peptide-HexNAc + 2H)$^{2+}$ (*m/z* 1,611). *N*-glycosylation at Asn117 is proven by the presence of b- and y-ion series derived from peptide T11b bearing HexNAc at Asn.

3.3.5. Heterogeneity Analysis at Each Glycosylation Site

The heterogeneity of glycosylation can be estimated from the *m/z* values and intensities of protonated ions in the integrated mass spectrum acquired at the elution positions of glycopeptides. The carbohydrate structure is deduced from the molecular masses of individual molecules.

Fig. 5A shows the integrated mass spectrum of glycopeptide T45-SA2 which is attached to disialylated bi- and tri-antennary oligosaccharides. Glycopeptides T45 were separated in order of sialylation (T45-SA0, -SA1, -SA2, and -SA3). **Fig. 5B** is the integrated mass spectrum of the other glycopeptides and suggests the linkage of monosialylated biantennary form to peptide T17.

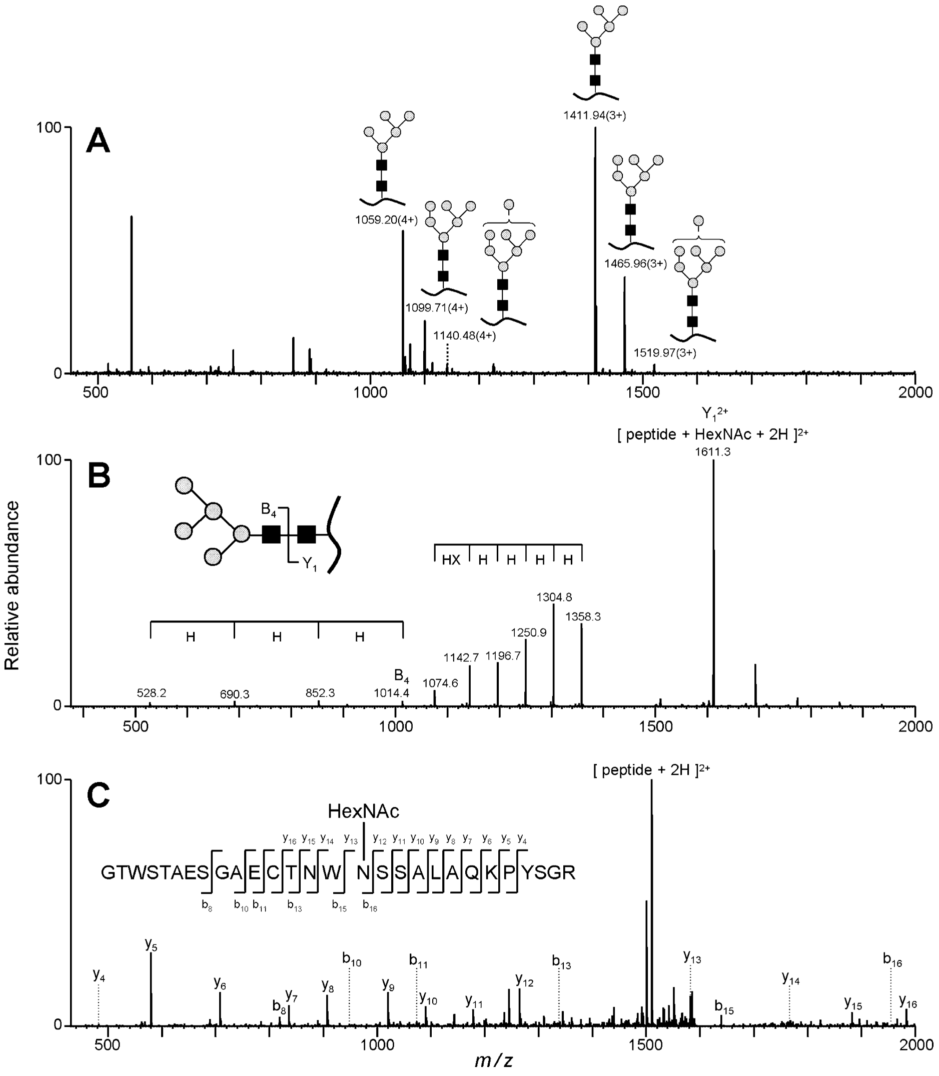

Fig. 4. MS~MS/MS/MS of glycopeptide T11b. **(A)** Mass spectrum acquired at 62.48 min. **(B)** MS/MS spectrum acquired from $(M + 3H)^{3+}$ (*m/z* 1411.94) as a precursor ion. Carbohydrate structure was deduced from the fragment pattern. **(C)** MS/MS/MS spectrum acquired from $(\text{Peptide-GlcNAc} + 2H)^{2+}$ (*m/z* 1611.3) as a precursor ion. Peptide sequence and glycosylation site were identified by the database search analysis.

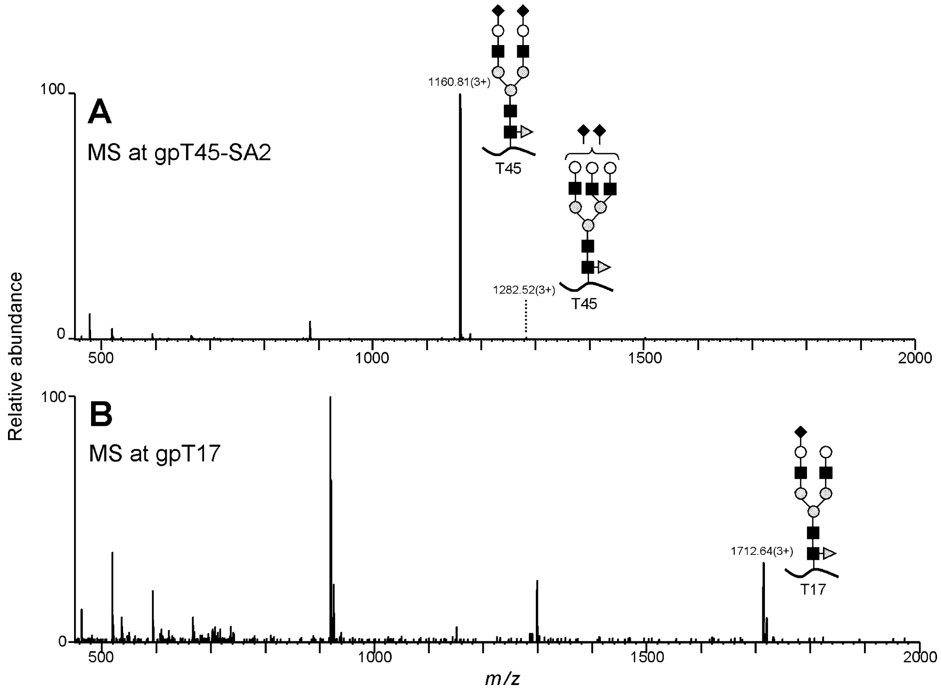

Fig. 5. Integrated mass spectra of glycopeptides derived from t-PA. **(A)** Integrated mass spectrum of glycopeptide T45-SA2 and structural assignment of glycopeptides. **(B)** Integrated mass spectrum of glycopeptide T17.

4. Notes

1. Enzyme specificity is as follows: Trypsin, Lys/Arg-↓-X (X≠Pro); Lys-C, Lys-↓-X; Glu-C, Glu (Asp)-↓-X; AspN, X-↓-Asp (Glu). Glu-C digests without cleavage of Asp-X at a 1:50 enzyme-to-substrate ratio at pH 8.0, and Asp-X can be hydrolyzed by Glu-C at a 1:4 enzyme-to-substrate ratio.
2. Typical flow rate against the column diameter is as follows: 0.3 mm, 5 μl/min; 0.2 mm, 3 μl/min; 0.1 mm, 0.5 μl/min; 0.075 mm, 0.3 μl/min.
3. It is recommended that a test run be conducted beforehand by using a standard sample, such as tryptic digested bovine serum albumin (BSA, commercially available. e.g., Michrom BioResources, Inc., Auburn, CA, USA). Database search analysis should be performed to check coverage of the amino acid sequence in BSA.
4. Positive ion mode is used for sequencing of both carbohydrate and peptide moieties. Negative ion mode is often used for the detection of highly sialylated or sulfated glycopeptides.

5. For the location of both *N*- and *O*-glycosylated peptides, monitoring of the oxonium ions at *m/z* 204 (HexNAc$^+$) produced in MS/MS is effective (10). However, in most cases, ion trap type mass spectrometer cannot detect small fragment ions, such as HexNAc$^+$, due to its low mass cut-off system. Hex-HexNAc$^+$ (*m/z* 366) and NeuAc-Hex-HexNAc$^+$ (*m/z* 657) can be used instead of HexNAc$^+$ (*m/z* 204). Monitoring HexNAc$^+$ (*m/z* 204) produced by in-source CID and the neutral loss of (*m/z* 162, 204, 291) by CID-MS/MS can be substituted for the locating the MS/MS spectra of glycopeptides ***(11, 12)***.

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Identification of Vitamin K-Dependent Proteins Using a Gla-Specific Monoclonal Antibody

Karin Hansson

1. Introduction

γ-Carboxyglutamic acid (Gla) is unique among naturally occurring amino acids in that it possesses two carboxyl groups on its side chain. The biosynthesis of Gla occurs post-translationally from specific glutamate (Glu) residues in vitamin K-dependent proteins and involves γ-glutamyl carboxylase, a resident enzyme of the endoplasmic reticulum ***(1–4)***. Gla was first discovered in the N-terminal so-called Gla domain of certain mammalian proteins involved in the haemostatic process ***(5,6,7)***. In these proteins the Gla residues chelate calcium ions and induce a conformational transition that endows the proteins with phospholipid membrane binding properties. Since its initial discovery Gla has been identified in a wide range of vertebrates including mammals, birds, reptiles and fish. In addition, Gla has been shown to be present in neurotoxic venom peptides of the invertebrate marine snails of genus *Conus* ***(8,9)***. These discoveries indicate that the role of this post-translational modification in blood coagulation represents only a subset of Gla functions in animal phyla and it appears that other Gla-containing proteins with novel functions remain to be identified in the future.

Several approaches have been developed for the qualitative and quantitative identification of Gla in proteins including isotopic labelling, specific stains, modified protein sequencing, mass spectrometry, ion exchange chromatography of alkaline hydrolysates, and gas or thin layer methodologies ***(5,10–15)***. However, most of these procedures are restricted as they require purified proteins or highly enriched preparations since most currently used methods for the measurement of Gla are fairly insensitive. Furthermore, Gla is particularly difficult to identify as it easily decarboxylates to Glu. Thus Gla residues are decarboxylated to Glu when heated under acid conditions, the step that normally precedes

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determination of amino acid composition. Alkaline hydrolysis is required to identify Gla and that is rarely done as several amino acids (but not Gla) are destroyed in this procedure. Also protein/peptide sequencing using the routine automated Edman degradation procedure does not provide straightforward identification of Gla. The phenylthiohydantoin (PTH) derivative of Gla is poorly extracted and it does not separate from other anionic amino acids during HPLC. Gla can routinely be identified during sequencing but this requires modification (methyl esterfication) of the peptide prior to sequencing to ensure adequate separation of Gla from other amino acids ***(12,16)***. Consequently, erroneous sequence assignments are likely unless the presence of Gla is expected and due precautions are taken. The approach described here circumvents these problems by identifying Gla-containing proteins prior to their analysis and allowing the protein to be traced easily during purification (*see* **Fig. 1**).

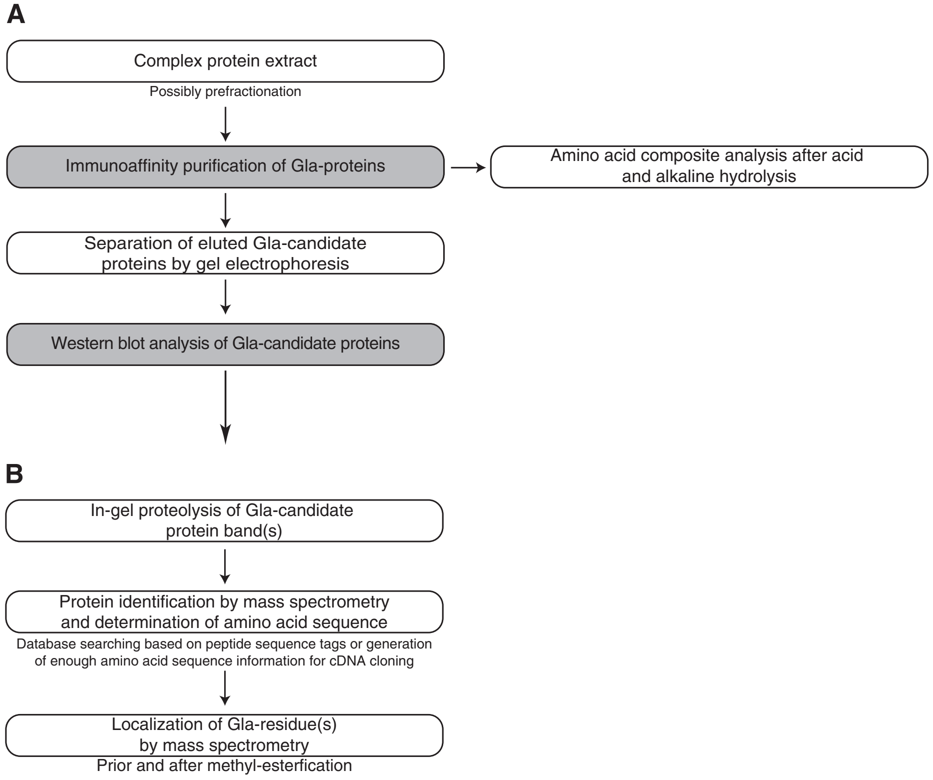
A
Complex protein extract
Possibly prefractionation
Immunoaffinity purification of Gla-proteins
Amino acid composite analysis after acid and alkaline hydrolysis
Separation of eluted Gla-candidate proteins by gel electrophoresis
Western blot analysis of Gla-candidate proteins
B
In-gel proteolysis of Gla-candidate protein band(s)
Protein identification by mass spectrometry and determination of amino acid sequence
Database searching based on peptide sequence tags or generation of enough amino acid sequence information for cDNA cloning
Localization of Gla-residue(s) by mass spectrometry
Prior and after methyl-esterfication

Fig. 1. Flow sheet showing the route for identification of Gla in proteins. Steps employing the Gla-specific antibody for the identification of vitamin K-dependent proteins are labeled with grey boxes. **(A)** Steps involved in the identification of Gla-candidate protein(s). **(B)** Steps involved in the localization of Gla-residue(s) in Gla-candidate protein(s).

The method depicted here is based on the development of mouse monoclonal antibodies that specifically recognize and bind Gla in proteins ***(17)***. The antibodies work well not only in Western blots but also with solution phase antigens, as has been shown by competitive immunoassays and surface plasmon resonance spectroscopy. Immobilized, the antibodies work well for affinity chromatography to purify Gla-containing proteins from complex protein extracts. The antibodies are unique and a highly functional tool that can be used to identify proteins containing one or more Gla residues ***(17–21)***. This tool in combination with modern mass spectrometry technique makes it possible to identify and localize Gla with high sensitivity in peptides and proteins.

2. Materials

2.1. Immunoaffinity Purification of Gla-Proteins

1. Mouse monoclonal antibody against Gla ***(17)*** (available from American Diagnostica Inc., CT).
2. Sulfolink coupling gel kit (Pierce Chemical Co., Rockford, IL).
3. Loading buffer: 20 m*M* Tris–HCl, 50 m*M* NaCl, 10 m*M* EDTA, pH 7.4 (*see* **Note 1**).
4. Column preservative: 0.05% (w/v) NaN_3 in Loading buffer.
5. Dialysis membrane (Spectra/Por 3, MW cutoff 3500, Spectrum Laboratories, Inc., CA) and 0.45-µm filter.
6. Wash buffer: 20 m*M* Tris–HCl, 300 m*M* NaCl, 10 m*M* EDTA, pH 7.4.
7. Elution buffer: 20 m*M* Tris–HCl, 50 m*M* $CaCl_2$, pH 7.4.
8. Protein storage buffer: 50 m*M* Tris–HCl, 100 m*M* NaCl, pH 8.0.

2.2. Separation of Eluted Gla-Candidate Proteins by Gel Electrophoresis

1. Separating buffer: 1.5 *M* Tris–HCl, pH 8.8.
2. Thirty percent acrylamide/Bis solution (37.5:1 with 2.6% C) (Bio-Rad Laboratories, Inc. CA) (*see* **Note 2**).
3. Twenty percent SDS.
4. N, N, N', N'-Tetramethyl-ethylendiamine (TEMED, Merck A/S, Oslo, Norway (*see* **Note 3**).
5. Ammonium persulfate: prepare 10% solution in water. Store at +4°C.
6. Stacking buffer: 0.5 *M* Tris–HCl, pH 6.8.
7. Electrophoresis buffer (5X): 250 m*M* Tris, 1.9 *M* glycine, 0.5% (w/v) SDS. Store at room temperature.
8. Sample buffer: 100 m*M* Tris–HCl pH 8.8, 0.015% (w/v) bromphenolblue, 36% (w/v) sucrose, 3% (w/v) SDS. Store in aliquots at −20°C.
9. 1 *M* dithiothreitol (DTT): prepare in water and immediately freeze in aliquots at −20°C.
10. Prestained molecular weight marker: MultiMark multi-colored standard (Invitrogen, Carlsbad, CA).

11. Silver stain reagents: fixation solution (50% (v/v) methanol, 5% (v/v) acetic acid), sensitizing solution (0.02% (w/v) sodium thiosulfate), staining solution (0.1% (v/v) silver nitrate), developing solution (2% (v/v) sodium carbonate), quenching solution (1% (v/v) acetic acid).

2.3. Western Blot Analysis of Gla-Candidate Proteins

1. Immobilone–P transfer membrane (Millipore, Bedford, MA) and 3 MM Chr chromatography filter paper (Whatman. Maidstone UK), two fiber pads.
2. Transfer buffer: 20 m*M* Tris, 150 m*M* glycine, 20% (v/v) ethanol.
3. Blocking buffer: 10% (w/v) skim milk powder in TBS.
4. Tris–buffered saline (TBS): Prepare 10X stock solution with 200 m*M* Tris–HCl, 1.54 *M* NaCl, pH 7.4. Dilute 100 mL with 900 mL water for use.
5. TBS with Tween (TBS–T): TBS supplemented with 0.2% (v/v) Tween 20.
6. Primary antibody: Mouse monoclonal antibody against Gla; M3B ***(17)*** (available from American Diagnostica Inc., CT).
7. Antibody dilution buffer: TBS–T containing 1% (w/v) fraction V bovine serum albumin (BSA).
8. Secondary antibody: Polyclonal rabbit anti-mouse IgG conjugated to alkaline-phosphatase (Dako A/S, Glostrup, Denmark).
9. 50 mg/mL BCIP (5-bromo-4-chloro-3-indolyl phosphate, Sigma chemical Co., MO) in DMSO. Prepare and store in the dark at +4°C.
10. 50 mg/ml NBT (2,2-di-*p*-nitrophenyl-5,5-diphenyl-3,3-[3,3-dimethoxy-4,4-diphenylene] ditetrazolium chloride (Nitro blue tetrazolium, Duchefa biochemie, Harleem, The Netherlands) in 50% (v/v) DMSO. Prepare and store in the dark at +4°C.
11. BCIP/NBT dilution buffer: 0.1 *M* Tris-HCl, 0.1 *M* NaCl, 5 m*M* $MgCl_2$, pH 9.0.
12. Stop solution: 0.02 *M* Tris-HCl, 5 m*M* EDTA, pH 8.0.

2.4. In-Gel Proteolysis of Gla-Candidate Protein Bands

1. Gel wash solution: 50% (v/v) acetonitrile.
2. Gel dehydration solution: 100% acetonitrile.
3. Reducing agent solution: 10 m*M* dithiothreitol (DTT), 0.1 *M* NH_4CO_3 pH 7.9.
4. Alkylating agent solution: 55 m*M* iodoacetic acid, 0.1 *M* NH_4HCO_3 pH 7.9.
5. Digestion buffer: 50 m*M* NH_4HCO_3 pH 7.9.
6. Endoproteinases: Trypsin (sequencing grade modified, Promega, Madison, WI), Asp-N (sequencing grade, Roche Diagnostics GmbH, Penzburg, Germany), Lys-C (sequencing grade, Roche Diagnostics GmbH, Penzburg, Germany).
7. Extraction solution: 5% (v/v) formic acid.

2.5. Mass Spectrometry

1. Micro-purification and desalting of peptides for mass spectrometry: $ZipTip_{C18}$ columns (Millipore Corporation, MA), Wetting solution: 50% (v/v) MeOH, Equilibration solution/Sample preparation solution/Wash solution: 1% (v/v) formic acid, Elution solution: 50% (v/v) MeOH, 1% (v/v) formic acid.

2. Quadropole-TOF hybrid mass spectrometer; we use a QSTAR pulsar-i quadropole-TOF tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (MDS Protana, Odense, Denmark).
3. Nanoelectrospray capillaries (New Objectives, MA).

2.6. Methyl-Esterfication to Confirm the Presence of Gla

1. Methylating reagent: Put 3 mL of methanol in a beaker. Place the beaker in a freezer and cool to −80°C. Take the beaker from the freezer and place in an ice bath and immediately add 510 µl acetyl chloride dropwise under constant agitation (*see* **Note 4**).
2. Nitrogen gas.

2.7. In-Solution Proteolysis of Purified Gla-Candidate Protein and HPLC Peptide Isolation

1. Denaturating solution: 6 *M* guanidine hydrochloride, 1 *M* Tris-HCl, 10 m*M* EDTA, pH 8.6.
2. Reducing agent: 1 *M* dithiothreitol (DTT).
3. Alkylating agent: 134 mg/ml iodoacetic acid in 0.5 *M* NaOH. Prepare fresh.
4. Quench solution: β-mercaptoethanol.
5. Dialysis membrane (Spectra/Por 3, MW cutoff 3500, Spectrum Laboratories, Inc., CA).
6. Twenty percent acetic acid.
7. Digestion buffer: 100 m*M* NH_4HCO_3 pH 7.9 (trypsin), 50 m*M* sodium phosphate buffer pH 8.0 (Asp-N), 25 m*M* Tris-HCl, 1 mM EDTA pH 8.5 (Lys-C).
8. Endoproteinases: Trypsin (sequencing grade modified, Promega, Madison, WI), Asp-N (sequencing grade, Roche Diagnostics GmbH, Penzburg, Germany), Lys-C (sequencing grade, Roche Diagnostics GmbH, Penzburg, Germany).
9. Peptide separation with reversed phase HPLC: 0.1% (v/v) trifluoroacetic acid (Buffer A), 0.1% (v/v) trifluoroacetic acid, acetonitrile (Buffer B). Degas both buffers before use.

2.8. Edman Degradation of Isolated Gla-Peptide(s)

1. Automatic sequence analyzer; we use a Procise 494 automatic sequencer (ABI, Foster City, CA) with standard reagents (ABI, Foster City, CA).
2. Biobrene Plus (Applied Biosystems, Foster City, CA).
3. Micro TFA filter (Perkin Elmer, Foster City, CA).
4. Cartridge seal (Applied Biosystems, Foster City, CA).
5. 0.1% (v/v) trifluoracetic acid.

2.9. Amino Acid Composite Analysis After Acid and Alkaline Hydrolysis

1. Dialysis membrane (Spectra/Por 3, MW cutoff 3500, Spectrum Laboratories, Inc., CA).
2. 20% acetic acid.

3. Hydrolysis tubes (10 × 100 mm, Schott Duran, Mainz, Germany). Wash tubes and incubate at 400°C overnight.
4. Alkaline hydrolysis solution: 4 *M* lithium hydroxide.
5. Acid hydrolysis solution: 6 *M* hydrochloric acid, 0.1% (w/v) phenol, 100 µ*M* norleucine. Prepare in water and store at +4°C.
6. Alkaline internal standard: 62 µ*M* β-carboxyglutamic acid (β-Gla). Prepare in water and store at +4°C.
7. Precipitation solution: 6.7 *M* phosphorous acid.
8. Sample dilution solution: Na-S high performance hydrolyzate sample dilution buffer (Beckman Coulter Inc., CA).
9. Standard solution for alkaline hydrolysis; 0.5 m*M* γ–carboxyglutamic acid (γ-Gla). Prepare in water and store at +4°C.
10. Standard solution for acid hydrolysis; Std amino acid standard for hydrolyzate analysis, CM-cysteine 3.4 nmol/µl, Beckman Coulter Inc., CA). Prepare in NaS and store at +4°C.
11. Automatic amino acid analyzer; we use a Beckman 6300 amino acid analyzer (Beckman Coulter, Inc., CA) and System gold software (Beckman Coulter Inc., CA).
12. High performance buffers Na-A, Na-B, Na-C, Na-D, Na-R, Na-S, Fluo-R (Beckman Coulter, Inc., CA).
13. Sodium high performance column (12 cm) (Beckman Coulter, Inc., CA).

3. Methods

As described in the introduction, the key to the specific and sensitive identification of vitamin K-dependent proteins in the method presented here is the recently developed mouse monoclonal antibodies that specifically recognize Gla in proteins. This unique tool permits the Gla-residue(s) to be traced during purification and analysis. The method description is mainly divided into two parts. The first part explains the identification and purification of Gla-proteins from biological fluids and tissues using the Gla-antibody for affinity chromatography and Western blot analysis (*see* **Subheadings 3.1** to **3.3; Fig. 1A**). The second part outlines the localization of Gla residues(s) in the sequence(s) by enzymatic digestion of the protein followed by peptide sequencing by mass spectrometry and/or Edman degradation prior and after methyl esterfication (*see* **Subheadings 3.4** to **3.9; Fig. 1B**).

3.1. Immunoaffinity Purification of Gla-proteins

1. Couple the Gla-specific monoclonal antibody M3B to Sulfolink coupling gel according to the manufacturer's instructions. These instructions assume the use of ~4 mg of antibody coupled to 2 mL of coupling gel.
2. Use the M3B-coupled resin to prepare an immunoaffinity column. Store the column in loading buffer containing 0.05% (w/v) NaN_3 at +4°C. Before use equilibrate the column with loading buffer at a flow rate of 3 mL/min.

3. Prepare the protein extract from the relevant biological fluid, tissue or organism and possibly pre-fractionate the material (*see* **Fig. 2A**). (It is beyond the scope of the present paper to discuss the protein extract preparation step in detail).
4. Dialyze the protein extract against loading buffer and filter by passage through a 0.45-µm filter. Load the filtrate onto the immunoaffinity column at a flow rate of 3 mL/min and monitor the A_{280} of the effluent (*see* **Fig. 2B**). After the A_{280} decreases to near the baseline wash/elute the column with wash buffer at 10 mL/min and elute subsequently the column with elution buffer. (Proteins containing a single Gla residue might bind loosely and elute with the wash buffer while proteins that enclose several Gla residues, for instance Gla-domain proteins elute with the elution buffer).

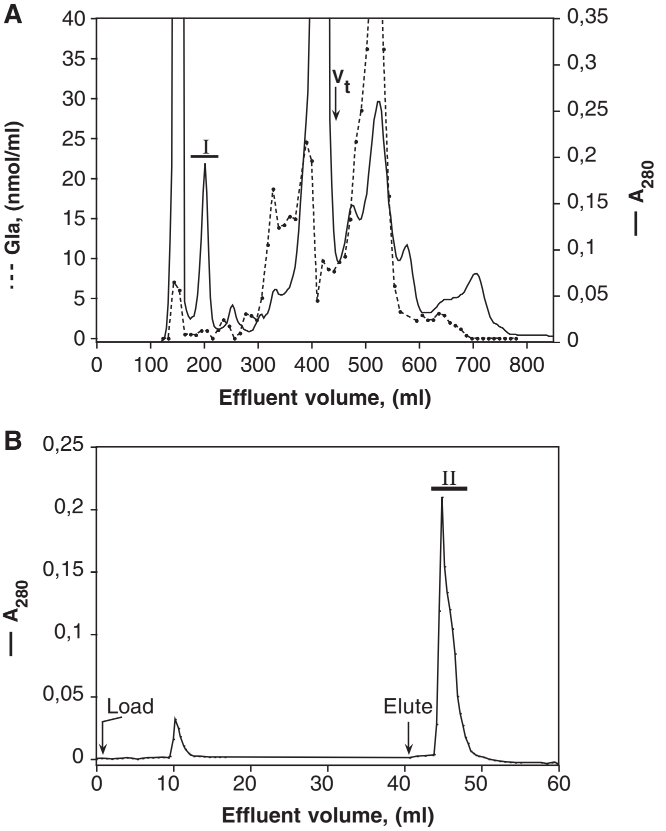

Fig. 2. Purification of a Gla-protein by immunoaffinity chromatography. **(A)** A complex protein extract prepared from the venom of the mollusc *Conus marmoreus* was pre-fractionated on a Sephadex G-50 Superfine gel filtration column. **(B)** The collected fractions denoted by a horizontal bar I (pool 1) were subjected to immunoaffinity chromatography on a column of monoclonal antibody M3B-coupled resin. The peak marked with a horizontal bar II contained the purified GlaCrisp protein containing a single Gla residue.

5. Dialyze the collected NaCl-eluted (wash-buffer) and/or $CaCl_2$-eluted (elution buffer) fractions extensively against protein storage buffer and store at −20°C.
6. Analyze the Gla-containing collected fractions by SDS-PAGE and Western blot analysis.

3.2. Separation of Eluted Gla-Candidate Proteins by Gel Electrophoresis

The protein(s) retained by affinity chromatography is subsequently analyzed by SDS-PAGE and Western blot employing the Gla-specific antibody. Prepare one gel for protein visualization and one gel for Western blot analysis.

1. This protocol is for two gels and assumes the use of a BioRad Mini-PROTEAN®3 Cell minigel system. Rinse the glass plates with distilled water and then with 95% ethanol and let them dry.
2. Prepare two 0.75-mm thick 12% gels by mixing 2.5 mL separating buffer with, 4.0 mL thirty percent acrylamide/Bis solution, 50 µL twenty percent SDS solution, 5.0 µL TEMED, 3.3 mL water, and 50 µL ammonium persulfate solution. Pour the gels, leaving space for stacking gel, and overlay immediately with water. Add the water slowly and evenly to avoid mixing. Allow the gels to polymerize for about 45 min.
3. Pour off the water and insert a piece of filter paper to dry the area in between the glass plates above the separating gels. Take care not to touch the surface of the gels.
4. Prepare the stacking gels by mixing 1.2 mL stacking buffer with, 0.6 mL thirty percent acrylamide/Bis solution, 25 µL twenty percent SDS solution, 5 µL TEMED, 3 mL water, and 25 µL ammonium persulfate solution. Pour the stacking gels until the top of the plates is reached and insert the desired combs. Allow the stacking gels to polymerize for 30 min.
5. Prepare the electrophoresis buffer by diluting 400 mL of the 5X electrophoresis buffer with 1600 mL of water in a cylinder. Cover with Para-Film and invert to mix.
6. Once the stacking gels have set, carefully remove the combs and use a 10-mL syringe fitted with a needle to wash the wells thoroughly with electrophoresis buffer.
7. Add the electrophoresis buffer to the inner chamber assembly and the lower buffer chamber.
8. Prepare the samples by mixing 20 µl (50 pmol; reduce the volume of the sample if necessary in a centrifugal vacuum concentrator) of each collected fraction with, 5 µl sample buffer, and 2 µl DTT solution (*see* **Note 5**). Incubate the samples for 5 min at 99°C. The samples are now ready for separation by SDS-PAGE.
9. Load the samples into the wells with a Hamilton syringe. Include one well for prestained molecular weight marker.
10. Run the gel at 150 V for 50 min or until the dye front is reaching the bottom of the gel.
11. Visualize the protein bands on one of the gels by silver staining (*see* **Fig. 3A**) (*see* **Note 6**). Fix the gel in fixation solution for 30 min. Rinse the gel with water (two changes, 2 min per change) and wash the gel with water for one hour on a shaking platform. Incubate the gel in sensitizing solution for 2 min with gentle shaking and rinse quickly with two changes of water (10 sec per change). Incubate the gel

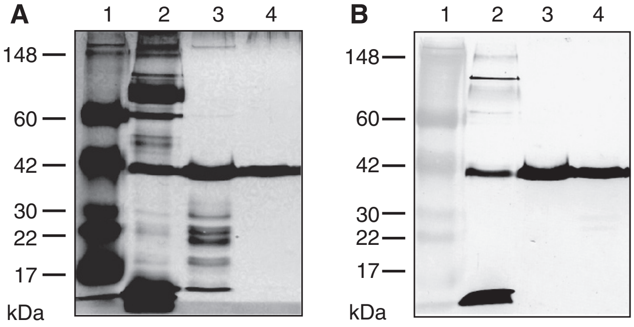

Fig. 3. Gel electrophoresis and Western blot analysis for the identification of Gla residues in proteins. **(A)** Protein samples from the purification steps of GlaCrisp (**Fig. 2**) were reduced, and resolved by 12% SDS-PAGE and silver stained. **(B)** The Gla-specific antibody M3B was employed to detect the Gla-containing components in the Western blot experiments. Key: lane 1, molecular weight marker; lane 2, crude *C. marmoreus* extract; lane 3, pool 1 from the Sephadex G-50 Superfine gel filtration column (**Fig. 2A**); lane 4, eluted fraction from the M3B immunoaffinity column (**Fig. 2B**).

in pre-chilled staining solution for 30 min at 4°C. Discard the staining solution and quickly rinse the gel with two changes of water (30 sec per change). Incubate the gel in developing solution at room temperature until adequate staining occurs. Quench staining by replacing the development solution with quenching solution. The gel can be stored in quenching buffer at +4°C.

3.3. Western Blot Analysis of Gla-Candidate Proteins

Transfer the protein bands on the second gel to an Immobilone–P transfer membrane electrophoretically.

1. Prepare a sheet of transfer membrane, cut just larger than the size of the separating gel by soaking it in 95% ethanol for a few seconds. Equilibrate the membrane, twelve pieces of 3 MM chromatography filter paper cut just larger than the transfer membrane, and two fiberpads in transfer buffer for a few minutes.
2. Remove the stacking gel from the separating gel with a razor blade.
3. Prepare the transfer sandwich by placing in the following order on the bottom anode electrode a fiber pad, six pieces of 3 MM chromatography filter paper, the transfer membrane, the gel, six pieces of 3 MM chromatography filter paper, a fiber pad, and on the top the cathode electrode. Avoid trapping of air bubbles between the gel and the transfer membrane.
4. Insert the transfer sandwich into the transfer tank. Fill the tank with transfer buffer. Attach the electrodes and electrophorese at 50 V overnight.
5. Once the transfer is complete take out the transfer sandwich of the tank and carefully disassemble it. Block the membrane in blocking buffer for 2 hrs at room temperature while shaking.

6. Remove the blocking buffer and rinse the membrane with TBS-T (four changes, 10 sec per change).
7. Incubate the membrane with 20 mL primary antibody M3B (10 μg/mL mouse monoclonal antibody against Gla diluted in antibody dilution buffer) for 2 hrs while shaking (*see* **Note 7**).
8. Wash the membrane with TBS-T (two changes with 10 sec per change, and four changes with 5 min per change).
9. Incubate the membrane with 20 mL secondary antibody (polyclonal rabbit anti-mouse IgG diluted 1:1000 in antibody dilution buffer) for 1 hr while shaking.
10. Wash the membrane with TBS-T (two changes with 10 sec per change, three changes with 5 min per change, and once for 10 min).
11. Incubate the membrane in 15 mL of BCIP/NBT substrate (prepare by mixing 100 μL 50 mg/mL BCIP solution with 50 μL 50 mg/ml NBT solution in BCIP/NBT dilution buffer) until adequate staining occurs (*see* **Fig. 3B**).
12. Stop development by replacing the BCIP/NBT substrate with stop solution.

3.4. In-Gel Proteolysis of Gla-Candidate Protein Bands

Wear gloves to reduce contamination with human keratins when preparing solutions and when handling samples. The described in-gel enzymatic digestion protocol is slightly modified from that described by Shevchenko et al. ***(22)***.

1. Rinse the silver stained gel with water two times for 10 min each.
2. Excise protein bands corresponding to immunoreactive protein bands in the Western blot analysis with a stainless steel cutting device. Cut as close to the edge of the band as possible to reduce the amount of background gel. Slice the gel band into small pieces (1 mm × 1 mm) and transfer them to a clean microcentrifuge tube.
3. Wash the gel with water (two times, 15 min each) and with gel wash solution (two times, 15 min each). The liquid volumes should be about five times the gel volume.
4. Remove the liquid and add 20 μL of gel dehydration solution. Incubate for 5 min until the gel pieces shrink and become white and stick together. Remove the gel dehydration solution.
5. Swell the gel pieces in 50 μL reducing agent solution and incubate for 1 h at 56°C to reduce disulfide bonds.
6. Cool the microcentrifuge tube to room temperature and remove the excess of reducing agent solution. Add quickly 50 μL alkylating agent solution and incubate for 30 min at room temperature in the dark.
7. Remove the alkylating agent solution and wash the gelpieces as described in step 3. Remove the liquid and add 20 μL of gel dehydration solution.
8. Rehydrate the gel pieces in enough digestion buffer containing 12.5 ng/μL trypsin to cover the gel pieces. Incubate on ice for 45 min. Add more digestion buffer if all liquid has been absorbed by the gel pieces. This protocol also applies to in-gel digestions with other commonly used endoproteinases such as Asp-N and Lys-C.
9. Remove the remaining liquid after 45 min and replace it with 20–25 μL of digestion buffer and incubate at 37°C overnight.

10. After overnight digestion spin the microcentrifuge tube shortly and extract the peptides from the gel pieces by adding 15 µL extraction solution and incubate for 15 min. Add an equal volume of acetonitrile and incubate for 15 min. Collect the supernatant. Repeat the extraction once and pool the extracts. Split the extract into two micocentrifuge tubes.
11. Dry down the pooled extracts using a vacuum centrifuge.

3.5. Protein Identification of In-Gel Digested Protein Bands by Mass Spectrometry

Localization of the Gla-residue(s) in the protein requires total coverage of the amino acid sequence followed by examination of the peptide masses, derived after enzymatic digestion, to target peptides that are carboxylated at their Glu residues. Mass spectrometric analysis is disturbed by the presence of salts and the sample must therefore be desalted. (It is beyond the scope of the present paper to discuss the identification of proteins by mass spectrometry analysis in detail). Analyze the extracted peptides by nano electrospray ionization (ESI) tandem mass spectrometry to obtain the entire amino acid sequence of the candidate Gla-protein.

1. Use a micro-purification and desalting microcolumn prior to the analysis of one half of the recovered peptides. Re-dissolve the dried peptides in 10 µL of sample preparation solution and desalt and concentrate the sample using a ZipTip$_{C18}$-column according to the manufacturers instructions. Elute the peptides in 1–5 µL elution solution.
2. Analyze the peptides by nano ESI tandem mass spectrometry where the peptides are first analyzed by full-scan MS and then by MS/MS. Program the software of the mass spectrometer to perform one scan to determine the peptide masses and then to sequence the two to four most abundant peptides.
3. Scan the spectra against a protein sequence database using a search algorithm such as Mascot or Sequest, which assigns peptide identifications based on matched criteria. If the protein is present in a protein sequence database and is identified the peptide assignments should be manually verified. If the protein proves to be unknown (i.e., not present in a sequence database) use the amino acid sequence tags obtained by *de novo* sequencing for subsequent construction of oligonucleotides to predict the complete amino acid sequence of the protein by cloning and cDNA sequencing. If necessary, sequence additional peptides manually by nano ESI MS/MS to generate enough sequence tags.

3.6. Localization of Gla-Residue(s) by Mass Spectrometry

1. Use the obtained amino acid sequence of the Gla-candidate protein to calculate the theoretical masses of the predicted peptides generated by in-gel digestion with the endoproteinase used.
2. Nano ESI mass spectrometry normally provides multiply charged peptide ions. Determine the mass of the peptides in the MS experiment described in **Subheading 3.5.**, by correcting the mass-to-charge values by the charge and the mass of added protons providing the charge.

3. Compare the observed peptide masses deduced from the ESI mass spectrometry analysis with the predicted theoretical peptide masses and target peptides that are carboxylated at their Glu residues. These peptides have a mass increase of 44 Da for each Gla residue as compared to the mass of the predicted Glu residue (*see* **Fig. 4A**). The ion signals in the MS spectrum corresponding to the Gla-containing peptides are typically accompanied by signals corresponding to loss of 44 Da caused by ejection of the γ-CO_2 by gas-phase decarboxylation or by loss of the γ-CO_2 during isolation and purification. To localize the peptide(s) containing Gla, it might be necessary to perform mass spectrometry analysis of peptides generated by endoproteinase in-gel digestions using two or more different enzymes, for example trypsin, Asp-N, and Lys-C.
4. Perform MS/MS fragmentation of the doubly and/or triply charged peptide ions corresponding to the Gla-peptide(s) (if this was not already performed in the MS/MS experiments described under **Subheading 3.5.**) to assign the position of the Gla residue(s) in the Gla-peptide(s).
5. Interpret the MS/MS fragment ion spectra and deduce the corresponding peptide sequences from the y and b ions. The peptidyl mass of Gla is 173 Da (*see* **Fig. 5**).

3.7. Methyl-Esterfication to Confirm the Presence of Gla

Confirm the presence of Gla-residue(s) in the protein by chemical derivatization of carboxyl groups in the peptides recovered from the in-gel digestion.

1. Add 50 µl of the methylating reagent to the other half of the dried peptides recovered after the in-gel digestion of the protein (*see* **Subheading 3.4.**).
2. Incubate for 2 hrs under nitrogen at room temperature.
3. Dry down the reaction mixture using a vacuum centrifuge.
4. Micropurify the derivatized peptides on a ZipTip_{C18} column as described in Subheading 3.5. and analyze the peptides by nano ESI mass spectrometry. Acquire spectra in both MS and MS/MS mode and correlate peptide molecular ions corresponding to the Gla-peptide(s) in the unmodified (*see* **Subheading 3.5.** and **3.6.**) and derivatized digests. Methanolic HCl adds one methyl group of 14 Da to Asp, Glu, S-carboxymethylated Cys, and to the C-terminus of the peptide and two methyl groups to Gla (*see* **Fig. 4B**).

3.8. In-Solution Proteolysis of Purified Gla-Candidate Protein and HPLC Peptide Isolation

If a pure Gla-protein(s) in sufficient amounts is retained by affinity chromatography (*see* **Subheading 3.1.**) it can be enzymatically digested and the generated peptides fractionated by reversed-phase HPLC to isolate the Gla-containing peptide(s).

1. Dissolve the protein (1 nmol) in 500 µl of denaturating solution in a microcentrifuge tube.
2. Add 10 µl of reducing agent to a final concentration of 20 m*M* and incubate at 37°C for 2 hrs (*see* **Note 8**).

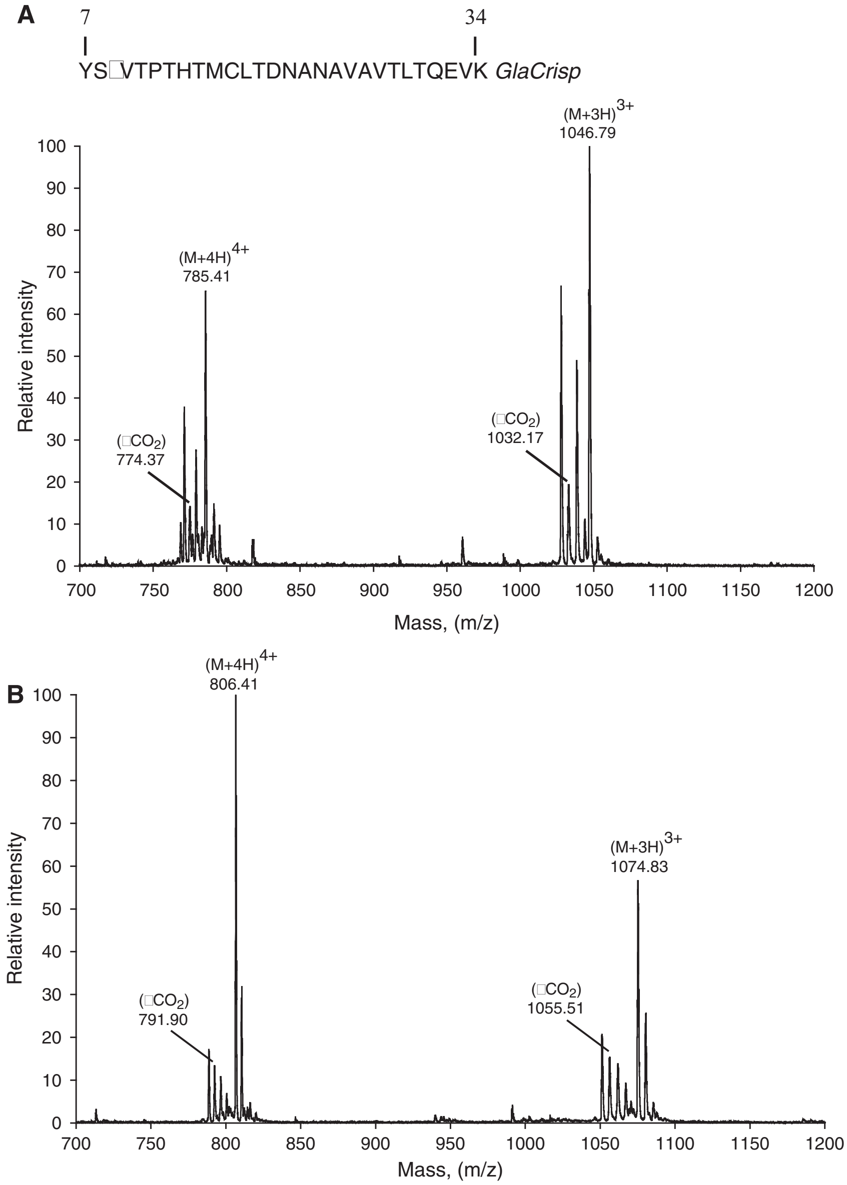

Fig. 4. Positive ion nano-ESI mass spectrometry analysis of a Gla-containing tryptic peptide fragment prior and after methyl-esterfication. The nanoESI-MS spectra of the S-carboxymethylated tryptic peptide fragment covering amino acid residues 7–34 of GlaCrisp prior **(A)** and after **(B)** methyl-esterfication. The ions at *m/z* 785.41 (4+), 1046.79 (3+), 806.41 (4+) and 1074.83 (3+) are accompanied by losses of 44 Da (corresponding to the mass of CO_2), which is consistent with a Gla residue in the peptide. After methyl esterfication an 84 Da increase in the monoisotopic molecular mass (corresponding to the methylation of all six carboxyl groups including the two at the Gla residue and the one at the C-terminus) was observed.

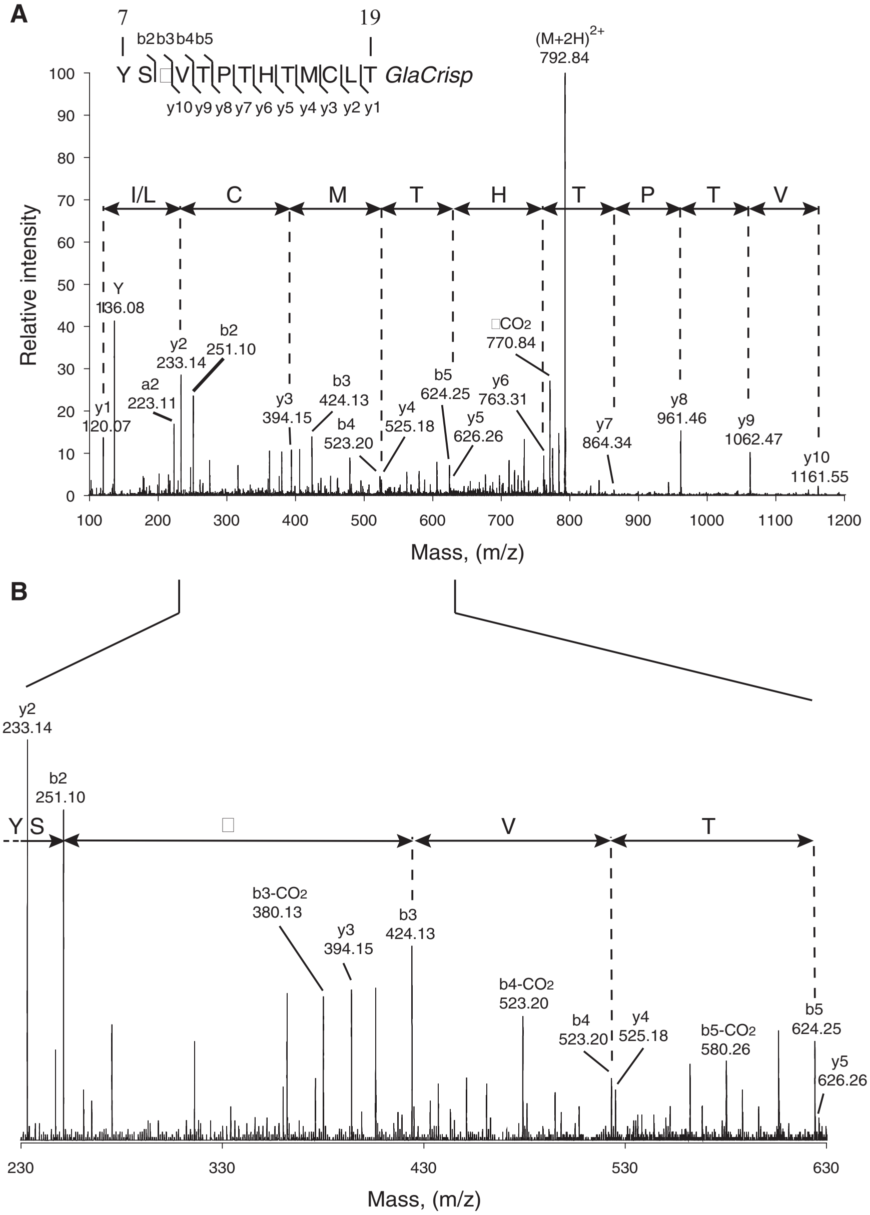

Fig. 5. Positive ion nanoESI-MS/MS analysis of the Gla-containing peptide fragment derived from digestions of GlaCrisp with trypsin and Asp-N. **(A)** The b and y ions according to the proposed sequence of the S-carboxymethylated internal peptide that covers amino acids 7–19 of GlaCrisp are labeled. **(B)** The *m/z* region from 230 to 630 has been expanded to show the b2, b3, b4, and b5 ions from which the N-terminal sequence of the peptide could be determined to localize the Gla residue.

3. Cool the microcentrifuge tube to room temperature and add 12.5 µl alkylating agent to a final concentration of 50 m*M*. Incubate in the dark at room temperature for 30 min.
4. Add 5 µl of quench solution to a final concentration of 1% (v/v).
5. Dialyze extensively against twenty percent acetic acid.
6. Dry down the reduced and alkylated protein using a vacuum centrifuge (*see* **Note 9**).
7. Add the endoproteinase in digestion buffer and incubate at 37°C for 18 hrs (*see* **Note 10**).
8. Dry down the digestion using a vacuum centrifuge.
9. Re-dissolve the dried peptides in 100 µl of buffer A and centrifuge for 1 min in a microcentrifuge at 10 000 rpm.
10. Remove the supernatant and inject onto an HPLC system equipped with a reversed-phase column (2.1 mm i.d.). After washing with buffer A elute the peptides using a linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 150 µl/min. Monitor the A_{214} and A_{280} of the effluent.
11. Analyze a small portion of the collected fractions of the HPLC separation by nano ESI tandem mass spectrometry, first by full-scan MS and then by MS/MS, to identify the fraction(s) containing the Gla-peptide(s) of the protein.

3.9. Edman Degradation of Isolated Gla-Peptide(s)

Verify the Gla residue(s) in the peptide sequence by Edman degradation. The current method for automated sequence analysis of Gla residues involves esterfication of the sample with methanolic HCl prior to sequencing.

1. Dry down two portions (100 pmol each) of the HPLC-isolated Gla-peptide(s) in microcentrifuge tubes using a vacuum centrifuge.
2. Perform methyl-esterfication as described in steps 1–3 in **Subheading 3.7.** of one of the portions of dried Gla-peptide(s).
3. Dry down the methyl-esterfied peptides using a vacuum centrifuge.
4. Re-dissolve the methylated and non-methylated portions of peptides in 0.1% trifluoracetic acid and load onto Biobrene Plus-pretreated micro TFA filters.
5. Analyze the Gla-peptide(s) both prior and after modification with methanolic HCl on an automatic sequence analyzer using the methods supplied by the manufacturer.
6. Evaluate the chromatograms and determine the amino acid sequence of the Gla-peptide(s). Compare the chromatograms of the peptide prior and after methylation. Methylation of the PTH derivatives of Glu and Asp causes a shift in the chromatographic elution time. The dimethyl ester of PTH Gla elutes between PTH proline and PTH methionine (*see* **Fig. 6**).

3.10. Amino Acid Composite Analysis After Acid and Alkaline Hydrolysis

Determine the amount of Gla in the isolated Gla-protein by performing amino acid analysis after acid and alkaline hydrolysis, respectively. Gla is extremely acid labile and is completely converted to glutamic acid during acid hydrolysis

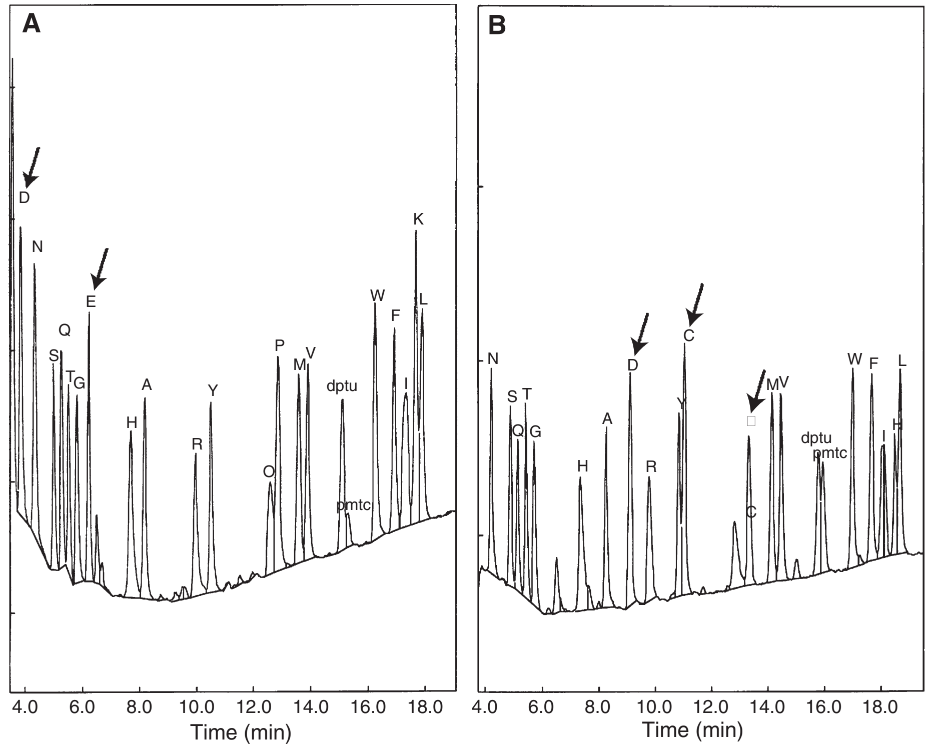

Fig. 6. C18 reversed phase HPLC chromatography of PTH amino acids. Separation of twenty-five picomoles of the standard mixture of PTH amino acids prior **(A)** and after **(B)** methyl-esterfication with methanolic HCl. The amino acid residues are designated by a one-letter code. γ denotes gamma-carboxyglutamic acid. DPTU and PMTC are by-products of the sequencing reaction and serve as useful reference peaks in the chromatogram. The arrows denote the PTH derivatives of Gla, Glu and Asp that show a shift in the chromatographic elution time caused by methylation. The absence of the Gla-derivative in the chromatogram prior to methyl esterfication is due to the low recovery from the extraction step prior to separation and inadequate separation from the other amino acid derivatives.

normally used for amino acid composite analysis. Alkaline hydrolysis provides quantitative release of Gla from peptide linkage without significant destruction.

1. Dialyze the purified protein (1 nmol as judged from A_{280}) suspected of containing Gla extensively against twenty percent acetic acid.
2. Divide the dialyzed protein into two hydrolysis tubes and dry down the samples using a vacuum centrifuge.
3. Add 100 µL of alkaline hydrolysis solution to one hydrolysis tube for alkaline hydrolysis. Add 100 µL of acid hydrolysis solution to the other hydrolysis tube for acid hydrolysis.

4. Seal the hydrolysis tubes under vacuum with a torch.
5. Heat the sealed tubes at 110°C for 24 hours.
6. Etch and crack the tubes at the top after hydrolysis.
7. Dry down the samples in a vacuum centrifuge.
8. For the alkaline hydrolysate: Add 100 μL alkaline internal standard and mix on a vortex for 15 sec. Add 20 μL precipitation solution and mix the sample on a vortex until a white precipitate is formed. Centrifuge the sample for 15 min at 10000 rpm and collect the clear supernatant. For the acid hydrolysate: Add 100 μL sample dilution solution.
9. For the alkaline hydrolysate: Mix 25 μL of the recovered supernatant with 35 μL of sample dilution solution and transfer to sample coil. For the acid hydrolysate: Mix 35 μL of the sample with 35 μL of sample dilution solution and transfer to a sample coil.
10. Analyze the samples on an automatic amino acid analyzer using the methods supplied by the manufacturer.
11. Evaluate the chromatograms and determine the amount of Gla in the protein.

4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ-cm. This standard is referred to as water in the text.
2. Caution! Acrylamide and Bis are toxic in the monomeric form. Wear gloves to avoid skin contact.
3. TEMED is best stored at +4°C in a desiccator.
4. Caution! Put on safety goggles and gloves. The mixture may boil up instantly.
5. For many Gla-proteins we have noticed that reduction of disulphide bonds generates more intense Western blot signals employing the Gla-specific antibody. This is probably due to increased exposure of Gla residues to the environment.
6. This silver-staining protocol in which glutardialdehyde is omitted is compatible with mass spectrometric analysis ***(22)***.
7. The primary antibody can be re-used for up to ten times only adjustment being increased length of incubation at the developing step with the BCIP/NBT substrate.
8. Less DTT can be used, but a 20- to 100-fold molar excess over protein thiols is advised.
9. Drying should be performed in the same microcentrifuge tube that will be used for the enzymatic digestion.
10. The recommended amount of enzyme is for trypsin and Lys-C 1:100 and for Asp-N 1:200 of the protein by weight.

Acknowledgments

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The Identification of Protein S-Nitrosocysteine

Todd M. Greco, Sheryl L. Stamer, Daniel C. Liebler, and Harry Ischiropoulos

1. Introduction

Nitric oxide ($NO^{\bullet}$) bioactivity regulates cellular function in most major mammalian organ systems. NO signaling either through soluble guanylate cyclase activation and cGMP production, or S-nitrosylation, the modification of cysteine residues to form S-nitrosocysteine, are currently the two most well studied nitric oxide signaling pathways. The emergence of S-nitrosylation as an intricate participant in cellular signaling is evidenced by the identification of endogenously S-nitrosylated proteins, including the NMDA receptor ***(1)***, caspase-3 ***(2)***, GAPDH ***(3)***, and NF-κB ***(4)***, which all participate in signal transduction pathways that regulate the fine balance between cell survival and cell death. The identification of novel S-nitrosylated proteins has traditionally stemmed from the observation that nitric oxide could modulate a protein's function, e.g. its enzymatic activity, and that these effects were often recapitulated when thiol reactive compounds such N-ethylmaleimide (NEM) or organomercury derivatives such as *p*-choloromercuribenzoic acid (PCMB) were used. Moreover, if the observed effects of nitric oxide could be reversed by reducantants such as dithiothreitol (DTT), cysteine modification was suspected. Researchers would then undertake the task of mutational analysis to identify which critical cysteine residue(s) was responsible for the observed effects.

While these methods are still critically important for establishing the functional role of S-nitrosylation, the development of proteomic techniques, namely the biotin switch method has accelerated the pace with which endogenously and potentially S-nitrosylated proteins have been identified. Here we describe an extension of the original biotin switch method ***(3)*** that incorporates peptide affinity capture, tandem mass spectrometry (MS/MS), and peptide sequence mapping,

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permitting both the S-nitrosylated protein and the corresponding S-nitrosocysteine residue to be identified in a single experiment ***(5,6)***.

2. Materials

2.1. Cell Culture and Lysis

1. Dulbecco's Modified Eagle's Medium (DMEM, Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT).
2. Hydrochloric acid (0.5 *M*, HCl) containing 0.1 m*M* DTPA. Store at room temperature.
3. L-cysteine (1 *M*): Prepared fresh in 0.5 N hydrochloric acid/0.1 mM DTPA.
4. Sodium nitrite (1 *M*): Prepared fresh in water (*see* **Note 1**).
5. S-nitrosocysteine stock solution (CysNO, ~0.5 M): Prepared by mixing equimolar concentrations of L-cysteine solution (1 *M*) and sodium nitrite solution (1 *M*). Vortex immediately and incubate on ice for 5 min. Neutralize with 1 N NaOH to pH 7.2–7.4, immediately aliquot (20 uL) and store at −80°C. Aliquots are single use only. The concentration of each aliquot should be determined before use (*see* **Note 2**).
6. D-PBS-MC: Dulbecco's Phosphate Buffered Saline (D-PBS, Gibco) supplemented with 1 mM diethylenetriaminepentaacetic acid (DTPA) and 0.1 m*M* neocuproine.
7. TE solution: trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA) (0.02%) (Gibco).
8. Isolation buffer: 250 mM HEPES-NaOH, pH 7.7, containing 1 m*M* DTPA, 0.1 m*M* neocuproine. Store at room temperature. Prepare fresh every 2–3 months.
9. Methyl methanethiosulfonate (MMTS, 10 *M*) (Sigma, St. Louis, MO).
10. Lysis buffer: Prepared fresh from isolation buffer adjusted to contain 1% Triton X-100 and 1 m*M* MMTS (*see* **Note 3**).

2.2. Biotin Switch Assay: Derivitization of Protein S-nitrosocysteine (Pr-SNO)

1. SDS stock solution: Prepared at 25% (12.5 g in 50 mL final volume of water). Store at room temperature. CAUTION: Respiratory irritant, wear appropriate protective equipment.
2. Acetone (Fisher Scientific, Waltham, MA). Store at −20°C. CAUTION: Flammable.
3. Resuspension buffer: Prepared fresh from 1 part isolation buffer, 8.6 parts water, and 0.4 parts SDS stock solution.
4. Blocking buffer: Prepared fresh from isolation buffer adjusted to 2.5% SDS and 20 m*M* MMTS.
5. Biotin-HPDP (Pierce, Rockland, IL): Stock solution prepared at 20 m*M* in dimethylformamide (DMF). Aliquot and store at −20°C.
6. Labeling buffer: Adjust 25 m*M* HEPES-NaOH, pH 7.7, containing 1 m*M* DTPA amd 0.1 m*M* neocuproine (prepared as a stock) to contain 0.2 m*M* biotin-HPDP and 1% SDS immediately before use.
7. Sodium ascorbate solution: Prepared fresh at 0.5 *M* in water. Store on ice and protect from light.

8. Bicarbonate buffer: Freshly prepared at 100 mM ammonium bicarbonate containing 0.1% SDS.
9. Bradford reagent (Bio-rad) and BCA protein assay (Pierce).

2.3. SDS-PAGE and Western Blotting for Biotinylated Proteins

1. MOPS SDS running buffer (20X, Invitrogen)
2. NuPAGE Bis-Tris 4–2% mini gels (Invitrogen) (*see* **Note 4**).
3. XCell SureLock™ Mini-Cell (Invitrogen).
4. SeeBlue molecular weight marker (Invitrogen).
5. Nonreducing loading buffer (6X): 375 m*M* Tris-HCl, pH 6.8, 7.5% SDS, 50% glycerol, and 0.1% bromophenol blue (w/v).
6. Base buffer (10X): 0.25 *M* Tris base (do not adjust pH), 1.9 *M* glycine.
7. Transfer buffer (1X): Mix 700 mL water, 200 mL methanol, and 100 mL 10X base buffer.
8. Mini Trans-Blot cell (Biorad, Hercules, CA).
9. Immobilon-FL (PVDF) transfer membrane (Millipore) and chromatography paper (3 MM Chr, Whatman, Maidstone, UK).
10. Tris-buffered saline (TBS, 10X): 0.5 *M* Tris-HCl, pH 7.4, and 1.5 *M* NaCl.
11. Tween 20 (Biorad).
12. TBS-T wash buffer: Dilute 100 mL of 10X TBS with 900 mL water and adjust to 0.05% Tween 20.
13. Membrane blocking buffer: 5% (w/v) nonfat dry milk in 1X TBS.
14. Mouse antibiotin antibody (200 µg/mL, Spring Bioscience, Fremont, CA).
15. Primary antibody solution: Membrane blocking buffer adjusted to 0.05% Tween 20 and 0.1 ug/mL mouse antibiotin antibody.
16. Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NE) (*see* **Note 5**).
17. Alexa Fluor 680 antimouse IgG secondary antibody (2 mg/mL, Molecular Probes).
18. Secondary antibody solution: 1 part membrane blocking buffer, 4 parts TBS-t; adjust to 0.2 µg/mL secondary antibody. Protect from light.

2.4. Protein Digestion and Peptide Capture

1. Microcon YM-10 (10 kDa NMWL) filters (Millipore).
2. Trypsin gold, mass spectrometry grade (Promega, Madison, WI). Resuspend at 1 mg/mL in 50 m*M* acetic acid. Store at –20°C. Use within 5 freeze-thaw cycles.
3. Streptavidin-agarose suspension (Pierce).
4. Bicarbonate wash buffer: Prepared fresh at 1 *M* ammonium bicarbonate in water.
5. Formic acid, mass spectrometry grade (Sigma).
6. SpeedVac system.

2.5. Peptide Identification by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

1. HPLC-grade water.
2. Acetonitrile, HPLC grade.

3. Thermo LTQ two-dimensional ion trap mass spectrometer equipped with a micro electrospray source and a Thermo Surveyor pump and autosampler (Thermo Scientific, San Jose, CA).
4. Capillary LC column (100 µm I.D. × 110 mm) packed with Jupiter C18 resin (5 µm, Phenomenex, Torrance, CA).
5. HPLC solvent A: 0.1% formic acid in HPLC-grade water.
6. HPLC solvent B: 0.1% formic acid in HPLC-grade acetonitrile.
7. Data analysis software: SEQUEST (Bioworks software, Thermo Scientific) and Scaffold (Proteome Software, Portland, OR) (*see* **Note 6**).

3. Methods

Protein S-nitrosocysteine (S-nitrosothiols or Pr-SNOs) are labile post-translational modifications, which are sensitive to reduction by ultraviolet (UV) light, transition metals, and reducing agents such as beta-mercaptoethanol or dithiothreitol. As a result, identification of Pr-SNOs are often performed by the biotin switch method, which blocks existing free thiols, then selectively reduces Pr-SNOs to the corresponding thiols using ascorbate while concurrently labeling the newly generated thiols with a biotin-containing dithiopyridine derivative ***(3)***. The biotin moiety allows selective enrichment and identification of proteins that previously contained S-nitrosocysteine residues.

As an extension of this method, the protocol described here uses affinity enrichment of peptides rather than proteins, taking advantage of liquid chromatography tandem mass spectrometry (LC-MS/MS) to identify both the site of S-nitrosylation as well as the protein (**Fig. 1**). The detection of Pr-SNO is described for cell culture (**Subheading 3.1**); however, the method can be used for the identification of Pr-SNO from tissues ***(5,7)***. Importantly, rapid isolation and processing of starting material should always be performed under reduced lighting conditions in buffers containing metal chelators and in the absence of reducing compounds. This method enables the identification of S-nitrosocysteine-containing peptides starting from 1 mg of protein that contains between 0.1–.5 nmol Pr-SNO/mg of protein. These levels can be generated by exposing cells to NO donor compounds such as the family of NONOates ***(6,8,9)***. In contrast, for the identification of endogenous Pr-SNO, between 10–50 mg of initial protein is recommended (*see* **Note 7**).

Also of critical importance is the inclusion of appropriate negative controls to confirm that the sites of S-nitrosylation were derived from authentic S-nitrosocysteine residues. For example, authentic Pr-SNO sites should not be identified from control samples that have been exposed to UV light (**Subheading 3.2, step 2**) or from control samples in which ascorbate has been omitted (**Subheading 3.2, step 7**). Although not discussed in detail, the suppression of nitric oxide production by pharmacological NOS enzyme inhibitors, such as L-NAME, or by genetic NOS knockout mice models can be used as negative controls. As an

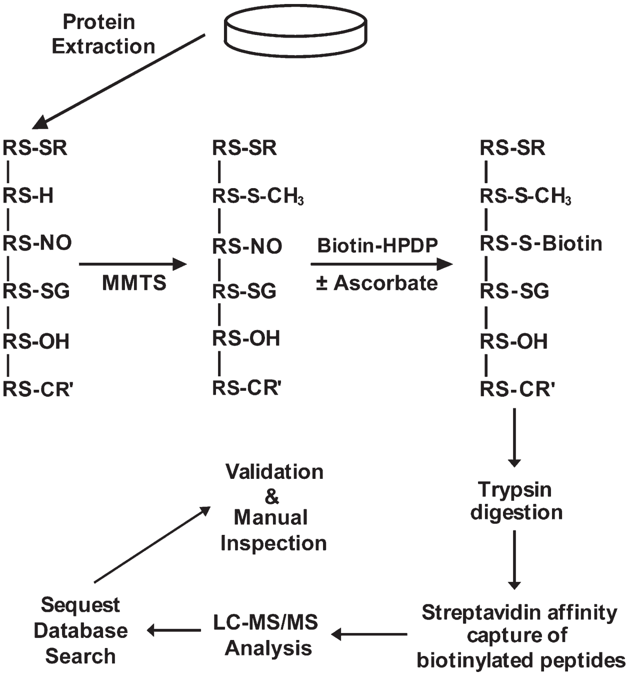
Protein Extraction
RS-SR
RS-H
RS-NO
RS-SG
RS-OH
RS-CR'
MMTS
RS-SR
RS-S-CH3
RS-NO
RS-SG
RS-OH
RS-CR'
Biotin-HPDP
± Ascorbate
RS-SR
RS-S-CH3
RS-S-Biotin
RS-SG
RS-OH
RS-CR'
Trypsin digestion
Streptavidin affinity capture of biotinylated peptides
LC-MS/MS Analysis
Sequest Database Search
Validation & Manual Inspection

Fig. 1. Scheme of the biotin switch method, modified for the site-specific identification of Pr-SNO residues by peptide affinity capture and tandem mass spectrometry. Depicted are multiple cysteine oxidation states that can occur in proteins: RS-H, reduced thiol; RS-SR, disulfide; RS-NO; S-nitrosated; RS-SG, S-glutathiolated; RS-OH, sulfenated; RS-CR', alkylated.

optional step (**Subheading 3.3**), a Western blotting protocol for immunodetection of biotinylated proteins is provided, which can be used to optimize the biotin switch assay before performing protein digestion, peptide capture, and LC-MS/MS analysis (**Subheadings 3.4** and **3.5**).

3.1. Cell Culture and Lysis

1. Cells are maintained in appropriate growth medium containing serum (e.g., DMEM containing 10% FBS). As the speed and efficiency of sample processing is critical for preservation of Pr-SNO, 2–3 treatment conditions are recommended. For example, a typical experiment consists of four cell culture flasks (175 cm^2) that are grown to 90–95% confluence and are either untreated (endogenous Pr-SNO) or treated with an NO donor (supplemented Pr-SNO), in duplicate.
2. To prepare cultures for treatment, aspirate serum-containing media and wash the cultures with warm serum-free media. Replace the media with either serum-free media alone or a serum-free media that has been freshly supplemented with 100 µM CysNO (*see* **Note 2**) and incubate cultures at 37°C for 20 min in the dark.

3. Transfer cultures to reduced lighting conditions and aspirate the treatment media. Wash with D-PBS-MC (1 × 10 mL) and then incubate with TE solution (5 mL) to detach the cells from the flask. After the cells have detached, inactivate TE solution by addition of ice-cold media containing 0.1% FBS (5 mL). Transfer cell suspensions from a single flask (10 mL) to one 15 mL centrifuge tube. Wash the culture flasks with ice-cold D-PBS-MC (1 × 5 mL) and combine with the cell suspensions. Pellet the cells by centrifugation (200 × g, 6 min, 4°C), then wash cell pellets with ice-cold D-PBS-MC (3 × 10 mL).
4. Add ice-cold lysis buffer (1 mL) to the cell pellets and triturate with a P200 pipette followed by gentle vortexing. Rotate crude cell lysates at 4°C in the dark for 30 min, followed by centrifugation (14,000 × g, 10 min, 4°C). Recover supernatant and rapidly determine the protein concentration using the Bradford reagent and bovine serum albumin as a standard. If necessary, dilute cell lysates to a final concentration of 0.8–1 mg/mL with lysis buffer (*see* **Note 8**).

3.2. Biotin Switch Assay: Derivitization of Pr-SNO

1. Unless otherwise stated, perform all steps on ice and either protected from direct sunlight or in the dark.
2. If a UV transilluminator or UV photolysis instrument is available, a negative control sample can be generated as UV light effectively reduces the S-NO bond (7, 10) (λ_{max} = 330–340 nm). For example, to use a UV transilluminator to generate Pr-SNO depleted samples, divide equal aliquots (2 × 1 mL) of cell lysates into Petri dishes. For negative control samples that are to be depleted, leave the dishes uncovered. For the aliquots where Pr-SNO is to be preserved, wrap the Petri dishes in aluminum foil. Place both sets of dishes in an ice trough and expose to UV light for 60 min by placing an inverted UV transilluminator over the dishes *(7)*.
3. Transfer lysates (1 mL) to 15 mL centrifuge tubes, washing the Petri dishes with an equal volume of lysis buffer to maximize protein recovery.
4. Precipitate proteins with 2 volumes (4 mL) of acetone (–20°C) and incubate at −20°C for 10 min, followed by centrifugation (3,000 × g, 10 min, 4°C). Discard the supernatant.
5. Resuspend the protein pellet in blocking buffer (1 mL) and incubate at 50°C for 30 min with frequent mixing.
6. Precipitate blocked proteins with 4 volumes (4 mL) of acetone (*see* **step 4**). Discard supernatant and solubilize pellet in resuspension buffer. Repeat acetone precipitation, then wash pellet with acetone (3 × 4 mL).
7. Resuspend pellets in labeling buffer (1 mL) and aliquot (2 × 0.5 mL) in 1.5 mL centrifuge tubes. To one aliquot, add 5 uL of sodium ascorbate solution (*see* **Notes 9** and **10**). Incubate at room temperature with gentle rotation.
8. Precipitate biotinylated proteins with 2 volumes (1 mL) acetone (*see* **step 4**). Discard supernatant, then wash pellet with acetone (3 × 1 mL). Resuspend protein in bicarbonate buffer (0.5 mL) (*see* **Note 11**). Samples no longer need to be protected from light. Estimate protein concentration using either the Bradford reagent or BCA assay.

3.3. SDS-PAGE and Western Blotting for Biotinylated Proteins

1. Prepare 1X MOPS running buffer (1 L) and assemble XCell Surelock Mini-cell system using a NuPAGE 4–12% Bis-Tris gel.
2. If necessary dilute samples to 1 mg/mL. Remove an aliquot of each sample (30 ul) and add 6X nonreducing sample buffer (6 μL).
3. Load 5 uL of SeeBlue molecular weight marker, followed by 30 uL of each sample per lane.
4. Electrophorese samples for at 150 V for 5 min, then at 200 V for 55 min at room temperature.
5. Transfer electrophoresed proteins to PVDF membrane using a Mini Trans-blot apparatus filled with 1X Transfer buffer. Using an ice cooler tank insert, transfer at 100 V for 1 h at 4°C.
6. After transfer, incubate PVDF membrane in membrane blocking buffer (25 mL) for at least 1 h at room temperature or overnight at 4°C under rotation.
7. Incubate PVDF membrane in primary antibody solution (10 mL) for 1 h at room temperature under rotation. Meanwhile, prepare 1X TBS-T wash buffer (0.5 L).
8. Discard primary antibody solution and wash PVDF membrane with TBS-T wash buffer (25 mL; 2 × 5 min, 2 × 10 min).
9. Incubate PVDF membrane with secondary antibody solution (10 mL) for 1 h at room temperature under rotation. Protect membrane from light.
10. Discard secondary antibody solution and wash PVDF membrane with TBS-T wash buffer (25 mL; 2 × 5 min, 1 × 10 min). Perform a final wash with TBS (25 mL; 1 × 10 min).
11. Scan PVDF membrane on the Odyssey Infrared Imaging system (*see* **Note 12**). A representative anti-biotin Western blot from CysNO-treated lysates that were processed by the biotin switch is shown (**Fig. 2**). Omission of ascorbate served as a negative control (**Fig. 2**, *lanes 1, 3, 5*).

3.4. Protein Digestion and Peptide Capture

1. Rinse Microcon YM-10 filters with 100% methanol (0.2 mL), and centrifuge (5,000 × g, RT). Wash with water (0.2 mL) and repeat centrifugation.
2. Immediately transfer biotinylated proteins (from **Subheading 3.2, step 8**) to prerinsed Microcon YM-10. Digest proteins by addition of trypsin (1:100 enzyme:protein) for 16 h at 37°C.
3. Centrifuge (5,000 × g, RT) until entire volume (~0.45 mL) has passed through the filter. Discard retentate and recover the filtrate containing a mixture of biotinylated and non-biotinylated peptides.
4. Remove 0.1 mL of streptavidin-agarose suspension from the stock (1:1 as supplied by Pierce) per mg of initial protein that was digested (across all samples). Wash streptavidin-agarose beads with 10 volumes of 0.1 M ammonium bicarbonate. Centrifuge (500 × g), discard supernatant, and resuspend beads in 0.1 M ammonium bicarbonate, preparing a homogenous 1:1 beads:bicarbonate suspension. Add 0.1 mL of streptavidin-agarose suspension per mg of initial protein that was digested (single sample) to each peptide mixture and incubate for 1 h at RT with gentle rotation.

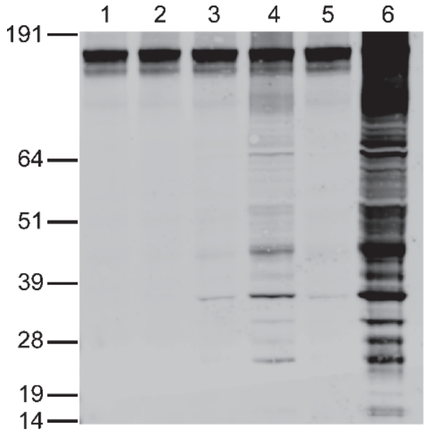

Fig. 2. Biotin switch Western blot for biotinylated proteins. Brain lysates were untreated (control) **(1,2)** or treated with 20 µM CysNO **(3–6)**, then processed by the biotin switch (**Subheading 3.2– 3.3**). All samples were incubated in labeling buffer containing biotin-HPDP. The detection of Pr-SNO is observed in both ascorbate-treated (**4**) and copper(II)/ascorbate-treated **(6)** samples (*see* **Note 9**). The absence of signal from the copper(II)/ascorbate-treated control sample **(2)** is due to the low levels of endogenous Pr-SNO (< 0.01 nmol/mg protein), which are below the limit of detection for this approach (~1 nmol/mg protein). Importantly, the omission of ascorbate from aliquots of **2**, **4**, and **6** displayed little to no signal (**1**, **3**, and **5**).

5. Centrifuge (500 × g, 1 min) and carefully aspirate the supernatant. Resuspend the beads in bicarbonate wash buffer (0.5 mL) and centrifuge (500 × g, 1 min). Repeat washing an additional 4 times, followed by 5 washes with water (0.5 mL).
6. Add 70% formic acid (100 µL) to the dry, washed streptavidin-agarose beads and incubate for 30 min at RT with gentle rotation to elute the biotinylated peptides. Centrifuge samples (1,000 × g, 5 min) and transfer the supernatant to clean microcentrifuge tubes. Repeat centrifugation and supernatant transfer to ensure agarose beads are removed from the sample.
7. Concentrate eluted peptides *in vacuo* to near dryness and resuspend in 0.1% formic acid (20 µL). If desired, peptides can be stored at −80°C for several months.
8. Transfer an aliquot of resuspended peptide mixture (10 µL) to an autosampler injection vial.

3.5. Peptide Identification by LC-MS/MS

Liquid chromatography coupled to electrospray ionization (ESI) tandem mass spectrometry (MS/MS) affords significantly improved sensitivity compared to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) or three-dimensional ion trap instruments. In particular, two-dimensional ion traps, such as the Thermo LTQ, are ideal for the identification of low abundance post-translational modifications from complex mixtures. This is due to its low femtomole limit of detection achieved by improved ion capacity, shorter duty cycle, and data-dependent MS^2 acquisition, as well as its ability to site-specifically map post-translational modifications via collision-induced dissociation (CID) peptide sequencing.

Yet this improved sensitivity, which typically results in the acquisition of 12,000–15,000 MS/MS spectra per sample, can be a double-edged sword. While the number of high quality, matched spectra is increased, the number of poor quality spectra resulting from the detection of low abundance peptides or simply noise is also increased. Therefore, this protocol employs a multistep analysis that uses both automated computational algorithms (SEQUEST and Scaffold) as well as manual inspection of MS/MS spectra to reduce false-positive peptide assignments (*see* **Subheading 3.5.2**).

3.5.1. LC-ESI/MS/MS Analysis

Liquid separation of peptides is achieved by reverse phase C18 using a Thermo Surveyor autosampler and pump, while ESI-MS/MS is performed by a Thermo LTQ ion trap mass spectrometer. Biotinylated peptides (7 µL) are subjected to reverse phase LC separation on a 0.1 × 110 mm fused silica capillary column fitted with a 60 mm frit (precolumn) packed with Jupiter C18 (5 µm particle size) using a flow rate of 0.7 µL/min. The mobile phase consisted of 0.1% formic acid in either HPLC grade water (A) or acetonitrile (B). Peptides are initially washed onto the column with 100% A for 10 min, followed 100 to 98% A for 5 min. Peptides are eluted with linear gradients of increasing solvent B from 2 to 25% B for 30 min, followed by 25% to 90% B for 15 min. As peptides are eluted from the capillary LC column they are subjected electrospray ionization (capillary voltage = 2 kV). Full scan (MS) spectra are acquired in positive ion mode from 400–2000 m/z, followed by data-dependent acquisition of MS/MS spectra on the four most intense ions from the MS scan.

3.5.2. Database Searching and Validation of Peptide Assignments

MS/MS spectra are extracted from the raw files using SEQUEST. SEQUEST output files are generated by searching raw spectra against theoretical MS/MS spectra generated from the *in silico* trypsinolysis of protein databases, such as the NCBI nr or IPI sequence databases, with a peptide mass tolerance of 2.5 amu and a fragment ion tolerance of 1.0 amu (*see* **Note 6**). Cysteine modification by methyl methanethiosulfonate (+46 atomic mass units) and by biotin-HPDP (+428 atomic mass units) are specified as variable modifications.

To compare SEQUEST sequence-to-spectrum assignments across several MS/MS experiments, SEQUEST output files are loaded into Scaffold. Only peptide assignments containing a biotin-HPDP-modified cysteine (+428 amu) are considered (*see* **Note 13**). Peptides assignments are first filtered by the following

SEQUEST threshold scores: $X_C \geq 2.5$ for doubly charged and ≥ 3.5 for triply charged ions; RSp ≤ 5; preliminary score (Sp) ≥ 350. Assignments that satisfy these criteria and are identified in at least 2 out of 3 independent experiments are manually reviewed ***(11)***. A peptide assignment with below threshold scores and/or marginal spectrum quality is accepted if it exhibits a pattern of fragmentation and relative ion peak intensity that is similar to an accepted peptide assignment present in another replicate. Shown in **Fig. 3** are representative MS/MS spectra that contain cysteine-modified biotin-HPDP, which were either accepted (**A**) or rejected (**B**). Finally, accepted peptide assignments are compared between controls (e.g. –ascorbate or +photolysis) and experimental treatments. Only peptides assignments unique to the experimental group are considered as authentic Pr-SNO peptide identifications.

4. Notes

1. Unless stated otherwise, “water” should be of > 18 MΩ-cm resistance and used for preparing all solutions.
2. After thawing the aliquot, the concentration is determined spectrophotometrically. First, blank a quartz cuvette against 999 uL of water, then add 1 uL of the stock solution, mix by inversion and read absorbance at 335 nm. Calculate the concentration of the stock solution by the following formula: $[\text{CysNO}] = (\text{Abs}_{335\,\text{nm}} \times 1000)/900\,\text{M}^{-1}\text{cm}^{-1}$. After determining the stock concentration store on ice and use within 30 min.
3. A moderate amount of MMTS is included in the lysis buffer to limit potential loss of Pr-SNO and/or artifactual transnitrosation chemistries that may occur during homogenization. However, if isolating tissue or cellular lysates for the purpose of post-lysis NO donor treatment, MMTS should be omitted.
4. Gel electrophoresis is described using NuPAGE Bis-Tris gradient mini gels, however, any standard SDS-PAGE system can be employed as long as the buffers (e.g., running and loading buffer) do not contain reducing agents. These gradient gels were used for their ability to efficiently resolve non-reduced protein samples as well as for their consistent transfer characteristics.

Fig. 3. (continued) This fragmentation pattern is consistent with the presence of glutamine residues (Q) in the sequence; therefore this assignment would be accepted. **(B)** Identified as the doubly-charged sequence $\text{TLEIEAC}_{428}\text{R}$ with an X_c of 2.59. Although this spectrum had a reasonable series of continuous y-ions, several of the most intense peaks (*arrows*) could not be ascribed to singly or doubly-charged fragment ions or to fragment ions containing neutral losses of either water. Without additional evidence, this assignment would be rejected.$^{2+}$ indicates a doubly-charged y- or b-ion fragment. * designates fragments that have lost ammonia, while $^{@}$ designates fragments that have lost water.

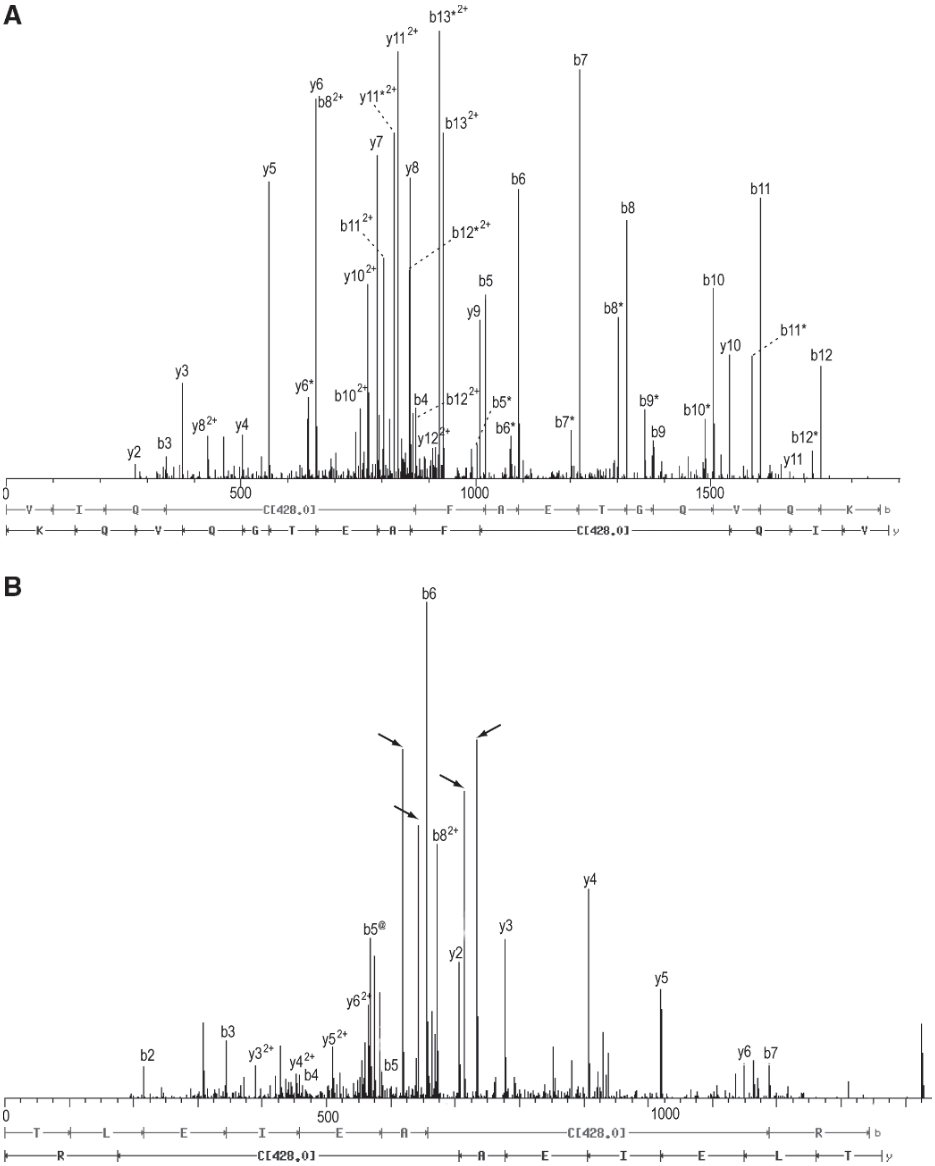

Fig. 3. Representative MS/MS spectra of Cys-biotin-HPDP labeled peptides. Both spectra passed the initial Sequest threshold scoring filter and were subjected to manual inspection (**Subheading 3.5.2**). **(A)** Identified as the doubly-charged sequence, VIQC$_{428}$FAETGQVQK, with an X$_c$ = 4.45. This spectrum contains well-matched peaks containing several y- and b-ions in series with most major peaks matched. Diagnostic pairs of b-ion fragments (b5/b5*-b12/b12*) are observed; corresponding to the expected fragment (b6, m/z = 1090.3) and the fragment resulting from a loss of ammonia (b6*, m/z = 1073.3).

5. Visualization of anti-biotin reactivity has been successfully performed with anti-mouse IgG conjugated to hRP; however, antimouse IgG conjugated to Alexa Fluor 680 allows near-infrared detection via the Odyssey Imaging system. For Western applications, this system provides linear range over 4–5 orders of magnitude as well as superior reproducibility compared to chemiluminescent detection.
6. SEQUEST evaluates peptide assignments primarily by cross correlation scores (X_c). This threshold scoring system allows defined cutoffs to be set for "correct" and "incorrect" peptides. However, the appropriate threshold will likely vary between different experimental conditions, such as instrument type, sample complexity, and protein database size. Also, SEQUEST scores do not inherently provide the probability that a peptide assignment is correct or incorrect. Currently, there are several statistical methods that can address these issues. For example, SEQUEST database searches can be performed against *in silco* digests of "reversed" databases, which contain the protein sequences in the normal orientation and an inverted orientation, where the protein sequences are listed from the C to N terminus. Peptide assignments to the reversed sequences are considered incorrect assignments and can be used to calculate the false positive (FP) or error rate for a particular experiment ***((12, 13))***. Appropriate SEQUEST threshold scores are chosen to obtain a desired FP rate. Usually ≤ 5% peptide FP rate is satisfactory for two-dimensional ion traps. As a complementary method for evaluating the quality of peptide assignments, the PeptideProphet and ProteinProphet algorithms utilize Bayesian statistics to determine the probability that an individual peptide assignment is correct ***(14,15)***. By assuming that correct and incorrect peptide assignments follow normal distributions, these algorithms in combination with manual inspection, provide an effective strategy for validating MS/MS peptide assignments generated from database search algorithms such as SEQUEST and Mascot. These algorithms are available as open source packages, implemented in the Trans-Proteomic Pipeline (http://tools.proteomecenter.org) or utilized within commercial software such as Scaffold (Proteome Software, Portland, OR).
7. In various rat tissues, endogenous Pr-SNOs are present between 1–10 pmol per mg of total protein ***(16)***. Similarly, in a cell culture model of murine macrophages (RAW 264.7), LPS-induced iNOS induction generated approximately 25 pmol of Pr-SNO per mg of total protein ***(17)***. For the current approach, between 0.1–0.5 nmol **total** Pr-SNO isolated from cells or tissue is the lowest recommended amount of modification to perform the analysis. These estimates suggest a starting protein amount of between 10 and 50 mg of protein per sample is appropriate. Given a single experimental

condition could be split into as many as 4 samples (±photolysis and ±ascorbate), obtaining this amount of starting material from cellular cultures is challenging. Currently, for cell culture experiments this usually requires samples to be processed by the biotin switch (**Subheading 3.2**) and then pooled from several experiments before proceeding to mass spectrometry analysis (**Subheading 3.4**). As technological advancements in MS sensitivity, mass accuracy, and sample processing are made these experiments will likely become more manageable.

8. Although protein yield from a nearly confluent 175 cm^2 cell culture flask will depend on the cell line used, a reasonable expectation is 2–5 mg. As described in the method, the yield from a single 175 cm^2 flask was 2 mg (primary human aortic smooth muscle cells).
9. Recent studies have suggested that not all S-NO bonds are equally susceptible to ascorbate reduction ***(18,19)***. Therefore, it is important to note that Pr-SNOs identified by this method likely reflect the subset, which are most susceptible to ascorbate reduction. As an alternative approach, our lab has observed significantly increased efficiency of Pr-SNO reduction using copper(II)/ascorbate (100 µ *M*/50 µ *M*) compared to ascorbate alone (5 mM) (**Fig. 2**, *Lane 3 vs 5*). Thus far we have not observed any nonspecificity due to Cu/Asc treatment by Western blotting ***(Lane 1 vs 2)*** or by LC-MS/MS **(data not shown)**.
10. Several studies have suggested that high concentrations of ascorbate (>10 m*M*) can generate nonspecific biotin-HPDP labeling through the reduction of disulfide bonds. This direct reaction is unlikely as even the one electron reduction of cystine by the dehydroascorbate radical is thermodynamically unfavorable ***(10)***. While direct mechanistic evidence for the ascorbate-dependent nonspecificity has not been demonstrated in the context of the biotin switch, it is likely that inadvertent exposure to UV light underlies this observation ***(10)***, highlighting the importance of protecting samples from sunlight exposure.
11. After bicarbonate resuspension, ensure that large clumps of the protein pellet are disrupted. It is normal if the solution appears cloudy; the protein will be digested efficiently by overnight trypsin incubation.
12. The default instrument settings as specified by the “membrane” preset are usually sufficient. Nonetheless, increased sensitivity and lower background has been observed if the membrane is first dehydrated in methanol, and then allowed to dry before scanning. This may require a lower intensity setting compared to the default method.
13. The specificity of peptide identification for biotin-HPDP labeled cysteines should be ≥90%. If this was not achieved, consider increasing the volume or number of washes before eluting the biotinylated peptides from the

streptavidin-agarose beads. Alternatively, manual inspection may reveal that these assignments are low quality spectra. In this case, the SEQUEST threshold filters should be adjusted and/or searching against a reverse database is recommended to ensure false-positive rates are ≤5% (*see* **Note 6**).

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Detection of Nitrotyrosine-Containing Proteins

Xianquan Zhan and Dominic M. Desiderio

1. Introduction

Tyrosine nitration in a protein results from the peroxynitrite anion ($ONOO^-$, generated from the chemical reaction of nitric oxide and superoxide anion) in a cell and from oxidative stress, and might be an important molecular event in the physiological and pathological processes of the human pituitary. The pituitary is main component of multiple hypothalamic–pituitary–target organ axis systems. Studies have indicated that nitric oxide (NO) ***(1)*** and nitric oxide synthase (NOS) participate in those axis systems ***(2–4)***, including luteinizing hormone (LH) ***(4–7)***, follicle-stimulating hormone (FSH) ***(5)***, prolactin (PRL) ***(8)***, growth hormone (GH) ***(9–11)***, and adrenocorticotropin (ACTH) ***(12)*** axis systems. We recently discovered several nitrotyrosine-containing proteins in human pituitary postmortem and nonfunctional adenoma tissues ***(1,13,14)***, and this chapter describes different methods for detecting nitrotyrosine containing proteins, using pituitary tissue as an example.

The detection and identification of an *in vivo* nitrotyrosine-containing protein is very challenging. Commercial antinitrotyrosine polyclonal/monoclonal antibodies, combined with different technologies such as enzyme-linked immunosorbent assay (ELISA) ***(15,16)***, one/two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D/2-D SDS-PAGE)-based Western blot assay ***(13,14,17,18)***, and immunoprecipitation ***(14)***, are effective methods to measure a nitrotyrsoine level, and to isolate, enrich, and detect an endogenous nitroprotein. ELISA has been extensively used to analyze redox production ***(15,16,19)***. ELISA is a classic method to measure the content of nitrotyrosine, and the ELISA assay kit is commercially available (Upstate Catalog No. 17–136). 1-D/2-D SDS-PAGE can separate and preferentially enrich nitroproteins based on the unique properties (molecular weight MW and electric point pI) of a protein. Western blot assay not only detects the

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tyrosine-containing protein, but also determines the relative level of nitrotyrosine. Modern tandem mass spectrometry ***(1,13,14)*** methods can determine the amino acid sequence of proteolytic fragment of a protein to locate the nitrotyrosine sites in a protein sequence to clarify the function and role of endogenous tyrosine nitration.

2. Materials

2.1. Tissue Homogenization

1. Liquid nitrogen, freezer (−80°C), polytron homogenizer (Model P710/35; Brinkmann Instruments, Westbury, NY), and Fisher sonicator (Model FS30H; Fisher, Pittsburgh, PA).
2. Sodium chloride (0.9%) solution in deionized destilled water (*see* **Note 1**).
3. Homogenizing buffer (10 ml): 2 M acetic acid and 0.1% (v/v) mercaptoethanol. Store at 4°C.
4. Bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL).

2.2. Enzyme-Linked immunosobent assay (ELISA)

1. Slide-A-Lyzer dialysis cassette 2K MWCO, 0.2–0.5 ml (Pierce Product number 66205).
2. Phosphate-buffered saline (PBS) (200 m*M*; 20 ×): 4.6 g NaH_2PO_4 (or 5.3 g NaH_2PO_4•H_2O), 23 g Na_2HPO_4, 175 g NaCl, and 0.2 g NaN_3 are dissolved in c.750 ml deionized destilled water. Adjust pH to 7.22. Add deionized destilled water to 1 liter. Store at room temperature. Dilute 50 ml of 20 × PBS with 950 ml deionized destilled water for use.
3. Bovine serum albumin (BSA), Fraction V (Sigma).
4. Tetranitromethane (Sigma Product Number T25003) (*see* **Note 2**).
5. 50 m*M* Tris-HCl, pH 8.0, 50 ml: 302.75 mg Tris-base is dissolved in 40 ml deionized destilled water, adjust pH value to 8.0 with hydrolytic chloride, and add deionized destilled water to 50 ml.
6. Mouse antinitrotyrosine monoclonal antibody, clone 1A6 (100 µg/100 µl Catalog number 05-233, Upstate Biotechnology, Lake Placid, NY). Store at −20°C.
7. Rabbit antimouse IgG conjugated with alkaline phosphatase. Store at 4°C.
8. Alkaline phosphatase substrate pNPP (Sigma Fast™ P-Nitrophenyl phosphate Tablet set: pNPP 1.0 mg/ml and Tris buffer 0.2 M) (Sigma). Store at 4°C.
9. Costar® EIA/RIA plate with 96-well flat bottom (Product number 122 06049; Corning Incorporated, Corning, NY).

2.3. 1-D Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (1-D SDS-PAGE)

1. Protein extraction buffer: 60 m*M* Tris-HCl pH 6.8, 2% SDS, 10 m*M* dithiothreitol, 100 µM butylated hydroxytoluene (BHT) (*see* **Note 3**), and 2 m*M* EDTA.
2. Resolving gel stock buffer: 1.5 *M* Tris-HCl, pH 8.8. Store at 4°C.

3. Stacking gel stock buffer: 0.5 *M* Tris-HCl, pH 6.8. Store at 4°C.
4. 30% Acrylamide/ 0.8% bisacrylamide solution (*see* **Note 4**).
5. 10% SDS: 10 g SDS is dissolved in 100 ml deionized destilled water.
6. N,N,N′,N′-tetramethyl-ethylenediamine (TEMED, Bio-Rad, Hercules, CA).
7. Ammonium persulfate (10%): 100 mg of ammonium persulfate is dissolved in 1 ml deionized destilled water prior to use (*see* **Note 5**).
8. 50% isobutanol in water: 50 ml isobutanol mix with 50 ml deionized destilled water. Store at room temperature. Mix well prior to use.
9. Electrophoresis running buffer (10×): 250 m*M* Tris, 1.92 *M* glycine, and 1% (w/v) SDS. Store at room temperature.
10. Sample loading buffer: 30% (v/v) glycerol, 2% SDS, 62.5 m*M* Tris pH 6.8, 50 m*M* DTT, 5 m*M* EDTA, 0.02% NaN_3. Store in 200 µl aliquots in −20°C.
11. Prestained molecular weight markers: All Blue Precision Plus ProteinTM Standards (Catalog No. 161-0373, Bio-Rad, Hercules, CA). Store in 20 µl aliquots at −20°C.

2.4. 2-D Gel Electrophoresis (2DGE)

1. Protein extracting buffer (1 ml): 7 *M* urea, 2 *M* thiourea, 4% (w/v) CHAPS, 100 m*M* DTT, 0.5% pharmalyte, and trace bromophenol blue (*see* **Notes 6** and **7**).
2. Rehydration buffer (1 ml): 7 *M* urea, 2 *M* thiourea, 4% (w/v) CHAPS, 60 m*M* DTT, 0.5% Pharmacia IPG buffer, and trace bromophenol blue (*see* **Note 7**).
3. Resolving-gel buffer stock (4×): 1.5 *M* Tris-HCl, pH 8.8. Store at 4°C.
4. Reducing equilibration buffer (50 ml): 375 m*M* Tris-HCl pH 8.8, 6 *M* urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2% DTT, and trace bromophenol blue (*see* **Note 8**).
5. Alkylation equilibration buffer (50 ml): 375 m*M* Tris-HCl pH 8.8, 6 *M* urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% w/v iodoacetamide, and trace bromophenol blue (*see* **Note 8**).
6. 40% acrylamide/bisacrylamide stock solution (29:1): 40% w/v acrylamide, 1.38% w/v N, N′-methylenebisacrylamide (*see* **Note 4**) (Catalog No. 161-0146, Bio-Rad).
7. 10% Ammonium persulfate (3 ml) is made just prior to use (*see* **Note 5**).
8. SDS electrophoresis buffer (1 ×; 25 L): 25 m*M* Tris, 192 mM glycine, and 0.1% SDS. Store at room temperature. (*see* **Note 9**).
9. 1% Agarose sealing solution (100 ml) is made by heating until the agarose is completely dissolved and kept at 80°C prior to use. (*see* **Note 10**).

2.5. Western Blotting for Nitrotyrosine Based on 1-D SDS-PAGE

1. Transfer buffer (1 L): 25 m*M* Tris, 192 m*M* glycine, and 10% (v/v) methanol.
2. Polyvinylidene fluoride (PVDF) membrane: Immobilon-P transfer membrane (Catalog No. IPVH 081 00; Millipore, Bedford, MA) (*see* **Note 11**).
3. Mini Trans-blot filter paper (7.5 × 10 cm, Catalog No. 1703932; Bio-Rad).
4. Tris-buffered saline (TBS) stock solution (10×): 250 m*M* Tris-HCl pH 7.4, 1.37 *M* NaCl, 27 m*M* KCl. Dilute 100 ml with 900 ml deionized water for use.
5. Blocking solution: 1% (w/v) fraction V bovine serum albumin in TBS.

6. Primary antibody: mouse antinitrotyrosine monoclonal antibody, clone 1A6 (100 µg/100 µl Catalog number 05-233, Upstate Biotechnology, Lake Placid, NY). Dilute in 1:2000 with 3% BSA in TBS solution (0.5 µg Ab/mL) for use (*see* **Note 12**).
7. Secondary antibody: Sheep antimouse IgG conjugated with horse radish peroxidase (Catalog No. NXA931, Amersham). Dilute 1:7500 in 5% nonfat dry milk (NFDM) in TBS solution for use.
8. Enhanced chemiluminescent (ECL) reagents (Catalog No. RPN2106; Armersham Amersham Biosciences, Piscataway, NJ) and Bio-Max ML film (Kodak, Rochester, NY).

2.6. Western Blotting for Nitrotyrosine Based on Large-Format 2DGE

1. Gel equilibration buffer (1 L): 25 m*M* Tris and 192 m*M* glycine.
2. PVDF membrane equilibration buffer (1 L): 25 m*M* Tris, 192 m*M* glycine, and 20% (v/v) methanol.
3. Polyvinylidene fluoride (PVDF) membrane: Hybond-P 20 × 20 cm (Product No. RPN2020F; Amersham), or Immobilon-P transfer membrane (IPVH 20200; Millipore, Bedford, MA).
4. NovaBlot Electrode Filter Paper (Product No. 80-1105-19; Amersham).
5. Anode transfer buffer R (1 L): 3.63% (w/v) Tris, 20% (v/v) methanol, pH 10.4.
6. Anode transfer buffer S (1 L): 0.303% (w/v) Tris, 20% (v/v) methanol, pH 10.4.
7. Cathode transfer buffer T (1 L): 0.52% (w/v) 6-amino-n-hexanoic acid, 20% (v/v) methanol, pH 7.6).
8. Phosphate-buffered saline (PBS) (200 m*M*; 20 ×): 4.6 g NaH_2PO_4 (or 5.3 g $NaH_2PO_4 \bullet H_2O$), 23 g Na_2HPO_4, 175 g NaCl, and 0.2 g NaN_3 are dissolved in c.750 ml deionized destilled water, adjust pH to 7.22, add deionized destilled water to 1 liter. Store at room temperature.
9. PBS: 10 m*M* PBS. 50 ml of 20 × PBS is diluted with 950 ml deionized water.
10. PBST: 10 m*M* PBS, 0.2% (v/v) Tween-20, and 0.01% sodium azide.
11. 0.3% BSA/PBST: 0.3% w/v BSA, 10 m*M* PBS, 0.2% v/v Tween-20, and 0.01% w/v sodium azide.
12. Primary antibody: Rabbit anti-human nitrotyrosine antibody (Sigma, N0409, St. Louis, MO). Dilute (1:1000=v:v) in a 0.3% BSA/PBST solution (1 µg Ab/mL) for use.
13. Secondary antibody: goat antirabbit alkaline phosphase-conjugated IgG (Product No. 31340; Pierce, Rockford, IL). Dilute (1:5000 = v:v) in a 0.3% BSA/PBST solution for use.
14. Development reagent: 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (1-Step™ NBT/BCIP; Pierce, Rockford, IL).

2.7. Immunoprecipitation of Nitrotyrosine-Containing Protein

1. M-PER® mammalian protein extraction reagent (Pierce).
2. ImmunoPure® Immobilized Protein G plus (Pierce). Store at 4°C (*see* **Note 13**).

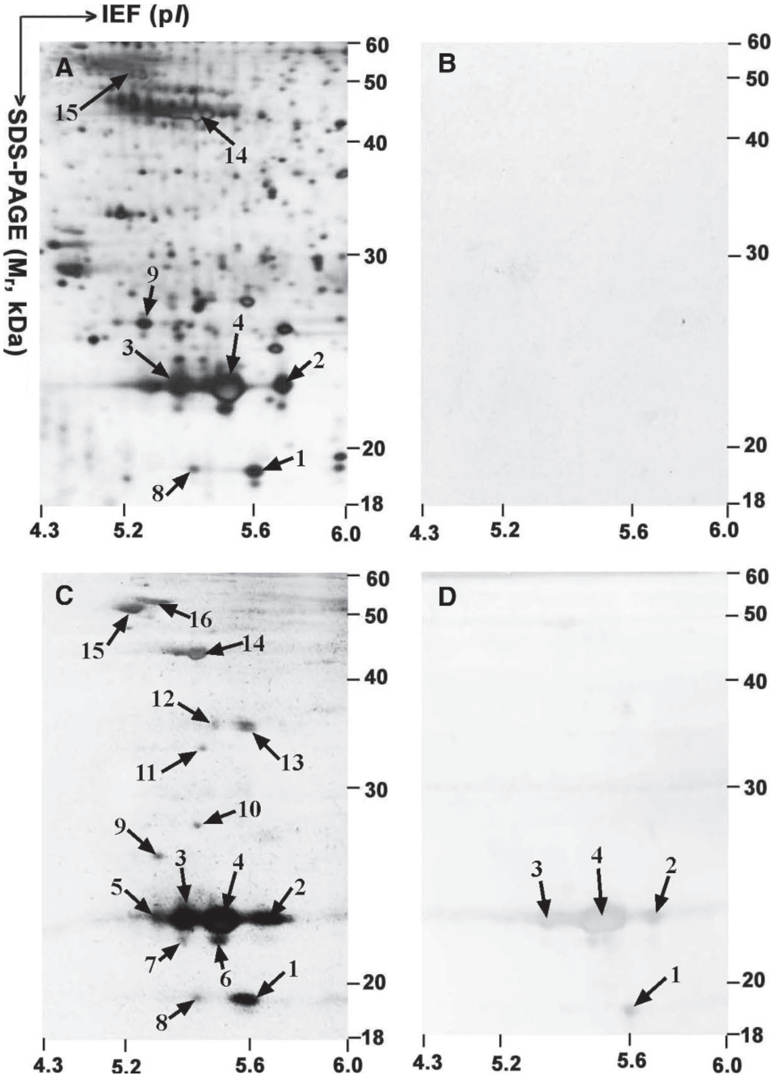
3. Binding/washing buffer: 140 m*M* NaCl, 8 m*M* sodium phosphate, 2 m*M* potassium phosphate, and 10 m*M* KCl, pH 7.4.
4. Rabbit Anti-nitrotyrosine polyclonal antibody (Chemicon® International, Inc.), or mouse anti-nitrotyrosine monoclonal antibody (Chemicon® International, Inc.).
5. Disuccinimidyl suberate (DSS), 2 mg/tube (Pierce) (*see* **Note 14**).
6. ImmunoPure® IgG Elution Buffer (Pierce), pH 2.8 contains primary amine.
7. Handee™ Spin Cup Columns (Pierce Product No. 69700).
8. Handee™ Microcentrifuge Tubes (Pierce Product No. 69715).
9. The pH-neutralized solution: 1 *M* Tris, pH 9.5.

2.8. Tandem Mass Spectrometry Characterization of Nitrotyrosine-Containing Protein

1. Sequencing grade modified trypsin (Catalog No. V511A, 5 × 20 µg aliquots; Promega). Store at −20°C (*see* **Note 15**)
2. Trypsin resuspension buffer (Promega Catalog No. V542A): 50 m*M* acetic acid.
3. 30 m*M* potassium ferricyanide (100 ml) is stored at 4°C.
4. 100 m*M* sodium thiosulfate (100 ml) is stored at 4°C.
5. 100 m*M* ammonium bicarbonate (100 ml) is stored at 4°C.
6. 50 m*M* ammonium bicarbonate (100 ml) is stored at 4°C.
7. 1% trifluoroacetic acid (TFA) (100 ml) is stored at 4°C.
8. 0.1% TFA (1 ml) is made prior to use.
9. 50% acetonitrile/0.1% TFA (1 ml) is made prior to use.
10. ZipTipC18 microcolumn (Millipore ZTC18S096, Bedford, MA).
11. 85% v/v acetonitrile/0.1% v/v TFA (1 ml) is made prior to use.
12. 2% actonitrile/0.5% acetic acid (1 ml) is made prior to use.

3. Methods

Tyrosine nitration in a protein is a relatively stable redox product that is produced due to oxidative/nitrative stress associated with physiological and pathology processes. The chemical structure of nitrotyrosine is a nitro (NO_2) group added to position 3 of the phenolic ring of a tyrosine residue. Commercially available anti-nitrotyrosine monoclonal and polyclonal antibodies enable one to determine the level of nitrotyrosine by ELISA (amount of nitrotyrosine per unit weight tissue), to detect the profile of nitrotyrosine-containing proteins by Western blotting based on 2DGE ***(1,13)*** (*see* **Fig. 1**) or 1-D SDS-PAGE ***(17,18)***, and to isolate and enrich nitroproteins by immunoprecipitation ***(14)*** and 2DGE Western analysis ***(1,13)***. For those isolated and enriched nitroproteins, tandem mass spectrometry is used to obtain each amino acid sequence and to identify each nitrotyrsoine-containing protein and nitration-sites ***(1,13,14)*** (*see* **Fig. 2**).


IEF (p*I*)
SDS-PAGE (M_r, kDa)
A
B
C
D
60
50
40
30
20
18
4.3
5.2
5.6
6.0
1
2
3
4
5
6
7
8
9
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11
12
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15
16

Fig. 1. Two-dimensional Western blot analysis of anti-3-nitrotyrosine proteins in a human pituitary (70 µg protein per 2-D gel). **(A)** Silver-stained image on a 2D gel before transfer of proteins to a PVDF membrane. **(B)** Silver-stained image on a 2D gel after transfer of proteins to a PVDF membrane. **(C)** Western blot image of anti-3-nitrotyrosine proteins (anti-3-nitrotyrosine antibodies + secondary antibody). **(D)** Negative control of Western blot to show the cross-reaction of the secondary antibody (only the secondary antibody; no anti-3-nitrotyrosine antibody). (Reproduced from ***ref. 1*** with permission from Elsevier Science).

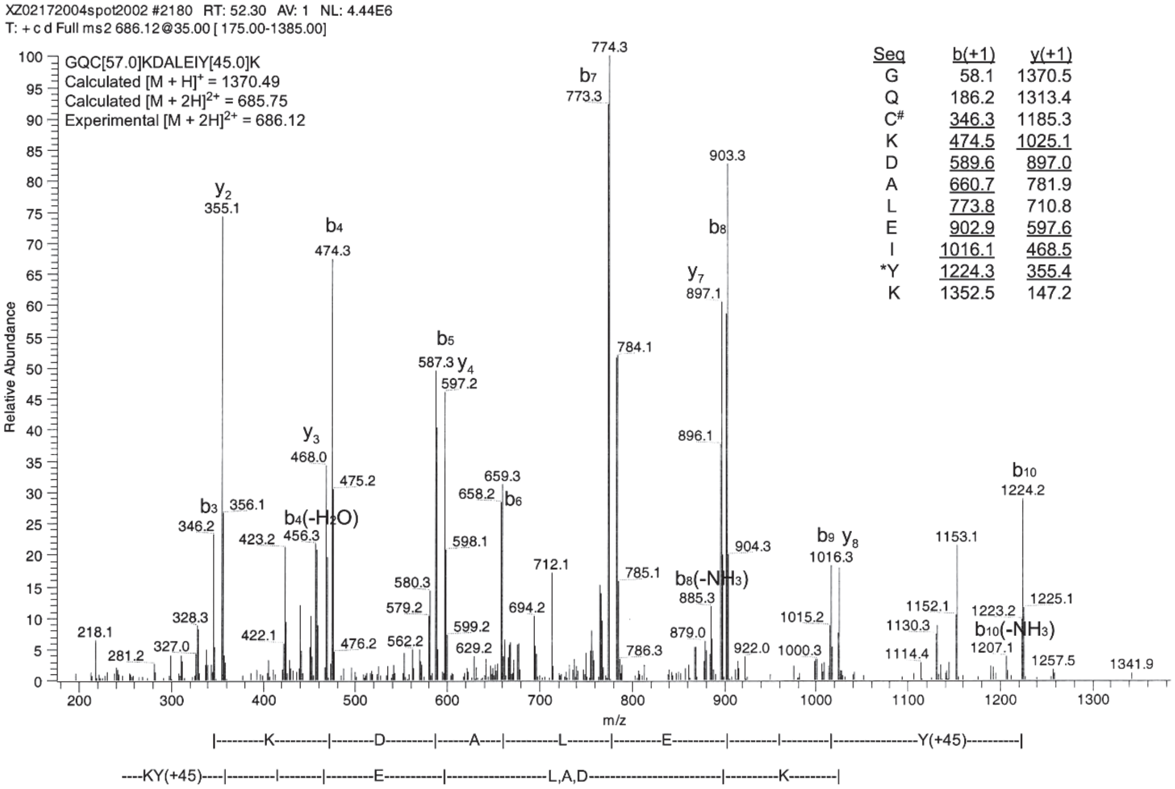

Fig. 2. SEQUEST **(top-right)** and de novo **(bottom)** analysis of an MS^2 spectrum of the precursor ion ($(M+2H)^{2+}$, at *m/z* = 686, RT = 52.3 min, and scan number 2180) for a nitrotyrosyl peptide (Tyr-237) ^{228}GQC$^{\#}$KDALEI*YK^{238} that contained 11 amino acids, and that derived from synaptosomal-associated protein. (Reproduced from ***ref. 1*** with permission from Elsevier Science).

3.1. Preparation of Samples

1. The human normal (control) pituitary tissue that is obtained from autopsy or the pituitary adenoma tissue that is obtained from neurosurgery is frozen immediately in liquid nitrogen, stored at – 80°C until processed.
2. Weigh the wet tissue: weigh an empty clean 15-ml tube (WT1), transfer the frozen tissue quickly and carefully, weigh the tube with tissue (WT2). The tissue weight = WT2-WT1.
3. Add 2 ml of 0.9% sodium chloride, and lightly shake to remove the blood on the surface of tissue. Repeat 3 times (*see* **Note 16**).
4. Add 10 ml homogenization buffer for c.0.5–0.6 g tissue, and homogenize fully (1 min × 10) with a Polytron homogenizer (13 000 rpm) in 4°C cold room. The homogenate is sonicated with a Fisher sonicator (Power setting = 5) for 20 s.
5. 1 ml of homogenate is centrifuged (10,000 g, 10 min). The supernatant is used for ELISA analysis to determine the nitrotyrosine concentration (see **step 1** in **Subheading 3.2**).
6. The remaining homogenate is lyophilized in 1 ml aliquots, and stored at – 80°C.
7. The total protein content in the lyophilized pituitary is measured with a bicinchoninic acid (BCA) protein assay reagent kit. (*see* **Note 17**).
   - (a) 280 µg of lyophilized pituitary sample is added into 264 µl solution that contains 8 *M* urea and 4% CHAPS. Stand for 2 h, sonicate for 5 min, rotate for 1 h, sonicate for 5 min, rotate for 1 h, centrifuge at 12,000 rpm for 20 min. The supernatant is used for the BCA analysis.
   - (b) Preparation of BSA standard solution: Dilute BSA standard (2 mg/ml) for the following dilution series: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 µg/ml.
   - (c) BCA working solution: 50 part of reagent A + 1 part of reagent B (50:1), mix fully prior to use.
   - (d) 0.1 ml sample or standard dilution is mixed with 2 ml BCA working solution (1:20). Incubate at 37°C for 30 min. Cool to room temperature (c.10 min). Measure the O.D. value ($A_{562\,nm}$) on the spectrophotometer.
   - (e) Use linear regression to make the standard line (BSA concentration vs. $A_{562\,nm}$), and obtain a regression equation that use $A_{562\,nm}$ to estimate the protein concentration. The regression equation is used to calculate the protein concentration of a lyophilized pituitary sample.

3.2. ELISA for Tissue Nitrotyrosine Level

1. The supernatant of 1 ml homogenate of pituitary tissue is dialyzed with a Slide-A-Lyzer dialysis cassette in 1000 ml PBS for 120 min. Change fresh PBS every 40 min.
2. Preparation of 20 µM standard nitrated BSA (50 ml): 66 mg BSA is dissolved in 50 ml of 50 m*M* Tris-HCl pH 8.0. Add 599 µl tetranitromethane into the above BSA solution, react for 5 min at room temperature. For removal of salt and tetranitromethane, the nitrated BSA solution is dialyzed with a Slide-A-Lyzer dialysis cassette in 1000 ml PBS for 120 min; change fresh PBS every 40 min. The dialyzed nitrated BSA is stored at – 80°C.

3. Make coating reagent: dilute 20 µ*M* nitrated BSA with PBS in 1:20 dilution.
4. Add 100 µl coating reagent per well into Costar® EIA/RIA plate with 96-well flat bottom. Incubate at 37°C for 1 h. Wash with 300 µl PBS/well for 3 times.
5. During coating, prepare the blocking solution: 1% Chicken ovalbumin in PBS.
6. Add 300 µl blocking solution per well, and incubate at 37°C for 1 h (Note: this step can be incubated longer). Wash with 300 µl PBS/well for 3 times.
7. During blocking, prepare 11 standard dilution series (0.4 dilution) of 20 µM (20000 pmol/ml) nitrated BSA: 20,000, 8,000, 3,200, 1,280, 512, 205, 82, 33, 13, 5, 2 pmol/ml, and each concentration 125 µl. Prepare 6 sample dilution series (0.2 dilution) of pituitary samples, and each concentration 125 µl. Meanwhile, prepare primary antibody: dilute mouse anti-nitrotyrosine monoclonal antibody in 1:2,000 dilution with 0.2% chicken ovalbumin in PBS.
8. Mix 125 µl of diluted primary antibody and 125 µl standard nitrated-BSA serial sample (or sample dilution series); incubate at 37°C for 1 h.
9. Add 100 µl of mixture above per well (duplicates), and shake at room temperature for 1 h. Wash with 300 µl PBS/well for 3 times.
10. Dilute secondary antibody (anti-mouse IgG-alkaline phosphatase) in 1:2000 dilution with 1% chicken ovalbumin in PBS. Add 100 µl diluted secondary antibody/well, shake at room temperature for 1 h. Wash with 300 µl PBS for 3 times.
11. Add alkaline phosphatase substrate pNPP solution (100 µl/well). Stand at room temperature until maximum absorbance reaches 0.6–1.0. Measure the absorbance at 405 nm and 655 nm.
12. Make the standard curve (log value of nitrated BSA concentration vs. O.D.) with the SigmaPlot software, and obtain a curve equation. Use sample O.D. value to calculate the nitrotyrosine concentration of pituitary sample (*see* **Note 18**). Convert the nitrotyrosine concentration to the nitrotyrosine amount per gram pituitary tissue.

3.3. 1-D SDS-PAGE and Western Blot for Nitrotyrosine Immunoactivity

1. Protein extraction for SDS-PAGE: An amount of lyophilized pituitary that contains 100 µg protein is dissolved in 100 µl protein extraction buffer, stand for 1 h, sonicate 20 s, rotate 1 h, sonicate 20 s. Centrifuge for 20 min at 13,000 × g. The supernatant is the extracted protein.
2. Bio-Rad Mini PROTEAN® Electrophoresis system is used; easily adaptable to other formats. The glass plates for gel is cleaned with the detergent; rinsed extensively with tap water, ethanol, distilled water, and ethanol; dried in a clean plastic rack for use. Install the long and short glass plates in the gel cassette (avoid the bottom leaking).
3. Preparation of a 1.5 mm thick, 12% resolving-gel: 4 ml of 30% acrylamide/0.8% bisacrylamide, 3.34 ml deionized water, 2.5 ml of 1.5 *M* Tris-HCl pH 8.8, and 100 µl of 10% SDS are mixed in a clean 50-ml tube. Add 50 µl of 10% ammonium persulfate and mix quickly, add 5 µl of TEMED, and mix quickly and fully. Pour the resolving gel solution into the assembled gel cassette (avoid generating

bubbles), leave 1.0–1.5 cm space for a stacking gel. Overlay the resolving gel with 1 ml of 50% isobutanol-water solution. Stand (room temperature) and avoid moving the gel cassette. The gel will be polymerized in 30–40 min. Remove the isobutanol and rinse with distilled water 3 times, and absorb the redundant water with KimWipe paper.

4. Preparation of a 4% stacking gel: 0.8 ml of 30% acrylamide/0.8% Bis, 3.6 ml deionized water, 1.5 ml 0.5 M Tris-HCl pH 6.8, and 60 µl 10% SDS are mixed in a clean 50-ml tube. Add 30 µl of 10% ammonium persulfate and mix quickly. Add 6 µl TEMED and mix quickly and fully. Pour the stacking gel solution onto the polymerized resolving gel up to the top-end of short glass plate. Insert quickly 1.5-mm thick and 10-well comb into the resolving gel. The stacking gel will be polymerized in 30–40 min.
5. Preparation of electrophoresis running buffer: dilute 100 ml of 10 × running buffer with 900 ml distilled water in a 1000-ml measuring cylinder.
6. Assemble the prepared gel cassette into the Bio-Rad Mini PROTEAN® electrophoresis cell. Add the running buffer into the upper and lower chambers of the gel unit. Let the electrophoresis running buffer cover the top-end of prepared gel. Remove gently and carefully the comb, and the running buffer will automatically fill in each well.
7. Mix 10 µl of the extracted protein and 20 µl of sample loading buffer in a 0.5-ml tube, and boil in 100°C water. Cool to room temperature. Load 30 µl of the prepared sample in a well. Meanwhile, load 5 µl of prestained molecular weight markers into another well.
8. Complete the assembly of the gel unit by covering the lid and connect to the power supply. The gel is run at constant 80 V for 10 min, then at constant 120 V. Stop electrophoresis when the dye front is 0.5 cm from the bottom of glass plate. After electrophoresis, the separated protein is visualized with development reagent, such as Gel-code blue (*see* **step 9**) and silver stain (*see* **step 21** in **Subheading 3.4**), or is transferred to PVDF membrane for Western blot analysis (*see* **step 10** and forward).
9. Disassemble the mini-gel unit. Remove the gel carefully, and avoid breaking the gel. Put gel in the clean flat tank, wash with distilled water (shake, 5 min). Replace with 100 ml Pierce Gel-Code blue reagent and shake gently for 1–2 h. Destain with distilled water until the gel band is clearly visible and the gel background is very low.
10. Disconnect the power supply and disassemble the mini-gel unit. Remove the gel, cut a small corner for labeling, and gently shake in transfer buffer for 10 min.
11. Preparation of PVDF membrane: cut a piece of PVDF membrane (6 × 9 cm) and put it in the 100% methanol and shake for 10 min. Replace methanol with distilled water to check quickly its hydrophilic side (water moves slowly on the hydrophilic side and fast on the hydrophobic side). Use pencil to write the date for label on the margin of the hydrophilic side. Replace water with transfer buffer and shake for at least 10 min.
12. Wet two Mini Tans-blot Filter papers (6 × 9 cm) and two clean fiber pads in the transfer buffer.

13. Assemble the transfer cassette: Put the black-side of the clean plastic transfer cassette down and open the cassette. Lay on one wet fiber pad onto the blackside. Lay on one sheet of wet filter paper on the wet fiber pad, and remove any bubbles. Put the gel on the filter paper and remove any bubbles. Put the PVDF membrane (the hydrophilic side down) on the gel and remove any bubbles. Put one sheet of wet filter paper on the PVDF and remove bubbles. Put one wet fiber pad on the filter paper. Close the transfer cassette.
14. The assembled transfer cassette is placed into the transfer tank with sufficient transfer buffer and a magnetic stir bar. The black side of the transfer cassette is toward the black side (cathode, -). Put into a flat box of cooling ice. The lid is put on the transfer tank and connected to the power supply. The transfer tank is covered with ice. Transfer is carried out at 320 mA constant for 2 h or at 300 V constant for 1 h.
15. After transfer, disconnect the power supply, disassemble the transfer cassette (Position the gel up and PVDF down), and use a pencil to draw a line around the gel on the hydrophilic side of PVDF for labeling. Remove the gel. Place the PVDF membrane into a block solution.
16. The PVDF membrane (protein-side up) is blocked in 1% BSA-TBS solution (gently shake for 1 h) (*see* **Note 19**). After block, the PVDF is washed two times with deionized water.
17. Add the primary antibody solution 30 ml (15 μl mouse anti-nitrotyrosine monoclonal antibody is diluted in 30 ml of 3% BSA in TBS solution), and incubate for 1 h at room temperature. Pour out the primary antibody solution. Wash with 100 ml of 0.2% Tween-20 in TBS for 10 min for 6 times.
18. Add the secondary antibody solution (6.7 μl antimouse IgG conjugated with horse radish peroxidase diluted in 50 ml of 5% nonfat dry milk in TBS), and incubate at room temperature for 1 h with gentle shaking. Pour out the secondary antibody solution. Wash with 100 ml of 0.2% Tween-20 in TBS for 10 min for 3 times. Wash with 100 ml of TBS for 10 min for 3 times. Rinse with deionized water for 4 times (*see* **Note 20**).
19. ECL visualization of the blotted PVDF membrane: Absorb the redundant water on the PVDF membrane between two sheets of filter paper. Place PVDF membrane on a clean glass plate (protein-side up) and cover with pre-prepared ECL reagent (4 ml ECL reagent A is mixed fully with 4 ml ECL reagent B prior to use). Incubate for 3–5 min. Dry the PVDF membrane between two sheets of filter paper. The PVDF membrane is placed between two transparent plastic sheets, and fixed in an X-ray film cassette. The remaining following steps are carried out in a dark room under the safe red light conditions.
20. Put a sheet of film on the transparent plastic sheet that contains the PVDF membrane and close the X-ray film cassette for a suitable exposure time, typically a few minutes. If the detected protein is very low, then expose for several hours to overnight. If the detected protein is very high, then expose for 10 s to 1 min. Remove the film and place it into an automatic image instrument to obtain the Western blotting image.

3.4. 2DGE and Western Blot for Nitrotyrosine Immunoactivity

1. Protein extraction for 2DGE: Weigh sufficient lyophilized pituitary sample to mix with 250 µl of the protein extracting buffer. The mixture is vortexted for 5 min, sonicated for 5 min, and rotated for 50 min. Add 110 µl of rehydration buffer. The mixture is sonicated for 5 min, rotate for 50 min, vortexed for 5 min, and centrifuged for 20 min at 15,000 g. The supernatant is the "protein sample solution" ***(20)***.
2. Rehydration of IPG strip (*see* **Note 21**): add 350 µl of the "protein sample solution" into a slot of the rehydration tray. Remove the plastic cover from the Dry IPG strip (do not touch gel-side). Place slowly and carefully strip gel-side-down on the "protein sample solution" in the rehydration tray; and distribute the "protein sample solution" evenly along the whole strip length under the IPG strip; avoid bubbles. Overlay strip with 3–4 ml of mineral oil. Rehydrate the strip overnight (c.18 h; room temperature).
3. The first-dimension, isoelectric focusing (IEF) is performed on an Amersham Multiphor II Electrophoresis system (*see* **steps 4–10**).
4. Assemble the Amersham Multiphor II instrument, connect the red bridging cable, and set the Multitemp water circulator to 20°C.
5. Add 6–7 ml of mineral oil to the center of the cooling plate. Position the Drystrip tray onto the mineral oil on the cooling plate (the red wire towards the rear); distribute mineral oil evenly between the Drystrip tray and the cooling plate (no bubbles). Insert red electrode wire (anode, +) into the rear receptacle; connect black electrode to the pin in the front.
6. Add 12 ml of mineral oil into the Drystrip tray. Place immobiline strip aligner (plastic sheet with grooves up) on top of the oil in the Drystip tray; distribute evenly the mineral oil between plastic sheet and the Drystrip tray (avoid bubbles; avoid getting mineral oil on top of plastic sheet); and line up grooves with the pattern on the cooling plate.
7. Take the rehydrated IPG strip out of the rehydration tray, rinse fully with deionized distilled water (*see* **Note 22**), drain. Place the IPG strip (gel-side-up; pointed end (acidic/anode) towards the rear) into a groove in the strip aligner. An immobiline strip aligner can analyze up to 12 IPG strips at a time.
8. Cut two electrode paper strips (length = 110 mm), and moisten each electrode strip with 0.5 ml of distilled water. Blot the excess water on the electrode paper strips with paper tissue to make them slightly damp. Place the two moistened electrode paper strips on the gel surface of those aligned IPG strips (one across the cathode and one across the anode edges) (*see* **Note 23**). Position the electrodes onto the electrode paper strips (Red electrode goes to the rear right, black to the front left).
9. Pour 70–80 ml of mineral oil to completely cover the IPG strips. Place the lid onto the Multiphor II instrument, and connect to Amersham EPS3500XL power supply. The IEF parameters are: Step 1: 100 V, 2 mA, 5 W, 1 min gradient; Step 2: 100 V, 2 mA, 5 W, 2 h set; Step 3: 500 V, 2 mA, 5 W, 1 min gradient; Step 4: 3,500 V, 2 mA, 5 W, 1.5 h gradient; and Step 5: 3,500 V, 2 mA, 5 W, 8 h set. The total time is 11 h 32 min, Vh c.31,000.

10. After IEF, disassemble the unit, remove IPG strips, gently blot off mineral oil, wrap loosely in plastic wrap, and store at −80°C (*see* **Note 24**).
11. The second-dimension, SDS-PAGE is performed on a Bio-Rad PROTEAN plus® Dodeca™ cell vertical electrophoresis system (*see* **steps 12–19**).
12. Cast 12% PAGE resolving gel with a Bio-Rad PROTEN-plus Multicasting chamber: Assemble the PROTEIN-plus Multicasting chamber system. For 12 gels, mix 180 ml of 40% w/v acrylamide/bisacrylamide stock solution (29:1), 150 ml of 1.5 M Tris-HCl pH 8.8, 270 ml deionized distilled water; de-gas with vacuum pump for 10 min. Add 3 ml of 10% ammonium persulfate, and 150 µl TEMED to the mixture solution (mix gently and avoid any bubbles). Gently pour the solution into holding chamber, and connect the holding chamber to the inlet port on multi-casting chamber. Place a gel comb in the first gel cassette. Elevate the holding chamber above the level of multi-casting chamber to fill the gel solution up to the level of the comb. Remove the comb, and overlay the gels with deionized distilled water immediately. Allow the gels to polymerize for >1 h (*see* **Note 25**).
13. Connect Dodeca cell tank to carboy that holds 25 L electrophoresis buffer to allow electrophoresis buffer to flow into the Dodeca buffer tank. Set the circulating water bath temperature to 15°C, and turn on the circulator at least 1 h prior to the actual run.
14. Take out the frozen focused IPG strips, stand at room temperature for 3 min, and put them (one in each slot) in the equilibrium tray (gel-side-up). Add c.4 ml of reducing equilibrium buffer, and rock gently for 10 min. Replace immediately the reducing equilibrium buffer with c.4 ml of alkylation equilibrium buffer for each slot, and rock gently for 10 min.
15. During equilibrium, discard the Multi-casting chamber. Remove the prepared gel plate, and rinse the top surface of gel with deionized distilled water three times. Absorb excess water on the top surface of gel with a KimWipe paper. Position the prepared gel plate on the gel-stander.
16. Remove the equilibrated IPG strip, rinse once in the electrophoresis buffer in a 20-cm length of cylinder; remove the redundant electrophoresis buffer on the surface of IPG strip. Position the IPG strip over the top of resolving gel, gel-side facing front, plastic back contacting the glass plate, the pointed-end of IPG strip to the left and the square end to the right. Load quickly *ca.* 3 ml of 1% agarose solution (c.80°C) onto the top of the resolving gel, push quickly the IPG strip into the un-polymerized agarose solution, and align the up-side of IPG strip onto the top of the shorter glass plate. Polymerize in c.10 min.
17. Use two hands to insert the prepared gel plate hinge side down (PROTEIN plus hinged spacer plate) near the bottom of the tank; the IPG stip-end of gel is positioned next to the cathode (black electrode card; -) so that the sample migrates toward the anode (red electrode card; +); and make sure that the gasket is flared out toward the electrode. Adjust the level of electrophoresis buffer to the middle of the top spacer (*see* **Note 26**).
18. Place the lid on the tank, and connect the pump tubing to the top of the lid. Set the Buffer Recirculation Pump to 100 and turn it on. Connect the Dodeca Cell to

the PowerPac 200 power supply. Electrophoresis is run under the conditions of constant voltage 200 V for 370 min.

19. After the electrophoresis, disassemble the Dodeca cell, take out the PROTEAN plus hinged spacer plate on the benchtop, short plate facing upward and the hinge to the left. Insert the gel releaser between the short and the long plate at the top right corner. Pull the gel releaser up, lift the short plate until the gel cassette is opened completely (180°). Separate gently the gel from the plate, and prevent the gel from tearing (*see* **Note 27**).
20. The separated protein on the 2DGE gel can be visualized with development reagent, such as silver stain (*see* **step 21**) and Gel-code blue (*see* **step 9** in **Subheading 3.3**), or transfer to PVDF membrane for Western blot analysis (*see* **step 22** and forward).
21. After electrophoresis, the gel is taken out, followed by silver staining: (1) Fix gel in 250 ml of (50% v/v methanol and 5% v/v acetic acid) (20 min); wash in 250 ml of 50% v/v methanol (10 min); wash in deionized water (10 min). (2) Sensitize the gel in 250 ml of 0.02% w/v sodium thiosulfate (1 min); wash with deionized water (1 min, 2 times). (3) Silver reaction (20 min) in 250 ml of the solution (0.1% w/v silver nitrate plus 200 µl 37% v/v formaldehyde prior to use); wash with deionized water (1 min, 2 times). (4) Develop in 250 ml of 3%w/v sodium carbonate with 100 µl 37% v/v formaldehyde (add prior to use) until the desired intensity of staining occurs (usually c.3 min). (5) Stop the development within 250 ml of 5% v/v acetic acid (10 min); wash the gel in deionized water (5 min). (6) Store the gel in 250 ml of 8.8% glycerol.
22. After electrophoresis, remove the 2D gel and orientate the 2D gel by removing the left-upper corner (acidic end), and soak the 2D gel in the gel equilibration buffer for at least 10 min.
23. Preparation of PVDF membrane: Cut a sheet of PVDF membrane to match the gel size (20 × 17.2 cm), and place into 100% methanol for 10 min, wash with destilled water for 5 min, and check its hydrophilic side (*see* **Note 28**). Use pencil to write the date on the margin of hydrophilic side of PVDF membrane. Equilibrate in the PVDF membrane equilibration buffer for at least 10 min (*see* **Note 29**).
24. The transfer is performed on an Amersham Multiphor II semi-dry electrotransfer system. Assemble the electroblotting cassette: (1) saturate anode electrode plate with deionized destilled water, and remove excess water with paper. Put it onto the buffer tank. (2) Immerse 6 filter papers in anode transfer buffer R, and place them carefully onto the anode plate. (3) Soak 3 filter papers in anode transfer buffer S, and place them carefully on the top of the first 6 filter papers. (4) Wet PVDF membrane in anode transfer buffer S for 30 s, and place PVDF (hydrophilic side up) onto the layer of second 3 sheets of filter papers. (5) Put the 2D gel onto the PVDF membrane. (6) Immerse 9 filtering papers in cathode transfer buffer T, and place them carefully on the top of the gel. (7) Saturate the cathode electrode plate with deionized distilled water, and remove excess water with a filter paper, place it on the 9 filtering papers. (8) Connect all units of the transfer system (*see* **Note 30**).

25. Close the lid and connect to the power supply (Amersham EPS 3501XL). Electrotransfer is performed at a constant current of 0.8 mA/cm^2 for 1 h 40 min. After transfer, remove the top filtering papers and draw a line around the 2D gel on the margin of PVDF membrane.
26. PVDF membrane (protein side up) is blocked in 100 ml of 0.3% BSA/PBST at room temperature for 60 min with gentle shaking. After blocking, the PVDF membrane is rinsed twice with water.
27. Add 100 ml of diluted primary antibody (100 µl rabbit anti-nitrotyrosine anbibody is diluted with 100 ml of 0.3% BSA/PBST). Incubate for 1 h at room temperature with gentle shaking. Pour out the primary antibody solution. Wash with 200 ml PBST with shaking (15 min; 4 ×). Rinse twice with deionized distilled water.
28. Add 100 ml of diluted secondary antibody (20 µl goat antirabbit alkaline phosphase-conjugated IgG is diluted in 100 ml of 0.3% BSA/PBST). Incubate at room temperature for 1 h with gentle shaking. Pour out the secondary antibody solution. Washing with 200 ml of PBST with shaking (15 min; 3 ×). Wash with 200 ml of PBS with shaking (15 min; 3 ×). Rinse 4 times with water (*see* **Note 20**).
29. The blot proteins are visualized by adding 1-Step™ NBT/BCIP substrate to cover the PVDF membrane (protein-side up) with gentle shaking until the desired color is obtained. Wash in deionized destilled water for 10 min. Dry the PVDF in the air or between two sheet of filter papers. Store between two leaves of transparent plastic sheet.
30. Scan the visualized PVDF membrane and the corresponding silver-stained 2D gel into a digitized image. Import the digitized image into the Bio-Rad PDQuest 2D gel image analysis software to generate the 2D gel spots and the PVDF blot spots. Match the immunopositive Western blotting spot to the corresponding silver-stained 2D gel spot (*see* **Figure 1**) (*see* **Note 31**).

3.5. Immunoprecipitation of Nitrotyrosine-Containing Proteins

1. Protein extraction for immunoprecipitation: A portion (e.g., c.62 mg wet weight tumor tissue) of pituitary tissue in 1.5-ml Eppendof tube is rinsed 3 times with binding/washing buffer to remove blood from the tissue surface. Add 600 µl of Pierce M-PER® mammalian protein extraction buffer that is compatible with immunoprecipitation (10:1 = buffer:tissue), votex 5 min, homogenize 5 min, sonicate 20 s, rotate 2 h, sonicate 20 s, and centrifuge at 13,000 g for 30 min. The supernatant is the extracted protein sample, which is transferred to a new tube. The protein content is measured with the Bio-Rad Bradford Protein Assay reagent.
2. Immunoprecipitation of nitrotyrosine-containing proteins is carried out with a Pierce Seize X mammalian immunoprecipitation kit. Equilibrate Immobilized Protein G, Anti-nitrotyrosine antibody, and binding/washing buffer to room temperature.
3. Gently swirl the bottle of ImmunoPure Immobilized Protein G beads to resuspend fully the beads, and add 400 µl of beads into a 0.5-ml Handee spin-cup column that is placed inside a Handee microcentrifuge tube. Centrifuge at 3,000 g for 1 min. Discard the flow-through. Wash the beads with 400 µl of binding/washing buffer

(invert tube 10 times, centrifuge 3,000 g, 1 min) for 2 times. Put the spin-cub into a new microcentrifuge tube.

4. Add 300 µl binding/washing buffer and 100 µl (= 100 µg) anti-nitrotyrosine antibody, and invert quickly the spin-cup to mix the antibody and beads. Incubate at room temperature for 1 h with gently rotating to allow antibody to bind the protein G. Centrifuge at 3,000 g for 1 min and discard the flow-through. Wash with 500 µl binding/washing buffer (invert 10 times, centrifuge at 3,000 g for 1 min) for 3 times. Put the spin-cup into a new microcentrifuge tube.
5. Dissolve 2 mg disuccinimidyl suberate (DSS) in 80 µl dimethyl sulfoxide (DMSO). Dilute 25 µl DSS with 400 µl binding/washing buffer, and add 425 µl diluted DSS solution to the washed beads with immobilized protein G antibodies. Invert the tube quickly and incubate for 30 min with gently rotating to allow the antibodies to crosslink with the immobilized protein G (*see* **Note 14**). Centrifuge at 3,000 g for 1 min, and discard the flow-through.
6. Wash the beads with crosslinked protein G-antibodies with 500 µl of elution buffer (pH 2.8) (invert 10 times, centrifuge at 3,000 g for 1 min) for 5 times. Put the spin-cup into a new microcentrifuge tube.
7. Wash the antibody-protein G-beads with 500 µl binding/washing buffeer (invert tube 10 times, centrifuge 3,000 g for 1 min) for 4 times.
8. Dilute the extracted pituitary protein samples with binding/washing buffer (at least 1:1 dilution). Add 500 µl of diluted sample to the prepared antibody-protein G-beads, mix quickly by inverting the tube. Incubate overnight (gently rocking, 19 h, 4°C) to bind nitrotyrosine-containing proteins with anti-nitrotyrosine antibodies. Centrifuge at 3,000 g for 1 min, and discard the flow-through.
9. Wash the beads with nitroprotein with 400 µl binding/washing buffer (invert tube 10 times, centrifuge 3,000 g for 1 min) for 3 times to remove any nonbound proteins.
10. Elute the bound nitroproteins with 200 µl elution buffer (pH 2.8) that contains a primary amine (gently mix well, centrifuge 3,000 g, 1 min) for 3 times. The eluates that contain nitroproteins are collected, add 10 µl of the pH-neutralized solution per 200 µl eluate. If necessary, the eluate may be dried partially to elevate the concentration of nitroprotein.

3.6. Tandem Mass Spectrometry Characterization of Nitrotyrosine-Containg Proteins

1. Trypsin digestion of immunoprecipitated nitroprotein: The immunoprecipitated protein is digested with trypsin (*see* **Note 32**). The procedure ***(14)*** is:
    (a) To a vial of sequencing grade-modified trypsin (20 µg, Promega, Madison, WI), add 5µl trypsin resuspension buffer that contains 50 m*M* acetic acid (pH 2.8) and 95 µl of 200 m*M* NH_4HCO_3 (pH 8.2), and mix fully.
    (b) Add 20 µl of the eluate that contains nitroproteins into a 0.5-ml siliconized tube (*see* **Note 33**).
    (c) Add 1µl of solution that contains 1 *M* Tris (pH 9.5), 100 m*M* dithiothreitol, and 100 m*M* iodoacetamide.

   (d) Add 25 µl trypsin solution and 54 µl ddH$_2$O, mix fully to form the enzyme digestion reaction system (100 µl, final concentration of NH$_4$HCO$_3$ = 50 m*M*, pH 8.1). Incubate overnight (37°C, 23 h).

2. Trypsin digestion of in-gel nitroprotein: Excise the protein spot that corresponded to the nitrotyrosine immunopositive spot from the 2-D gels, and digest with trypsin (*see* **Note 32**). The procedure ***(20)*** is:

   (a) Cut the silver-stained gel spots and place into a 1.5 mL siliconized tube (*see* **Note 33**). Wash 6 times with 500 µl of deionized water. Transfer the gel into another 1.5-mL siliconized tube, and mince it into several pieces (c.0.5–1 mm^3) with a pipette tip.
   (b) Pior to use, prepare the destaining solution by mixing 30 m*M* potassium ferricyanide with 100 m*M* sodium thiosulfate (1:1 v/v). Add 20 µl of fresh destaining solution to destain the gel pieces until the brownish color disappears (c.1–2 min). Remove the destaining solution, and wash 5–6 times the gel pieces with 20 µl water until the yellow color disappears.
   (c) Incubate the gel pieces for 20 min in 20 µl of 200 m*M* ammonium bicarbonate. Discard the buffer, and wash once the gel pieces with 20 µl water.
   (d) Dehydrate the gel pieces with 30 µl acetonitrile for 2 times until the gel pieces turn an opaque white. Dry in a vacuum centrifuge for 30 min at 30°C.
   (e) Add 100 µl of trypsin resuspension buffer to a vial of lyophilized trypsin powder (20 µg, 833 pmol) (Promega V5111), and mix fully. Dilute the trypsin solution (20 µl, 200 ng/µl) to 16 ng/µl with 230 µl of 50 mM ammonium bicarbonate.
   (f) Add 20–30 µl (0.32–0.48 µg) of the diluted trypsin solution to each dried gel piece. The gel piece should be covered by the digestion solution. Incubate (37°C, c.18–20 h), cool (4°C, 30 min).
   (g) Gently centrifuge for 10 s, sonicate in a water bath (30°C) for 5–6 min, and centrifuge at 12,000 g for 2 min. Transfer carefully the supernatant into a 0.5-ml siliconized tube.
   (h) Add 10–15 µl of 50 mM ammonium bicarbonate to the gel pieces to repeatedly extract (2 ×) tryptic peptides **(step 7)**. Combine all supernatants (the tryptic peptide mixture).

3. The tryptic peptide mixture from a 2-D gel spot or 20–40 µl tryptic peptide mixture from immunoprecipitated proteins is purified with a ZipTipC18 beads microcolumn (Millipore ZTC18S096, Bedford, MA). Set up the pipette to the 10-µl scale. Wash 5 times the beads with 10 µl acetonitrile, and 5 times with 10 µl of 50% acetonitrile. Equilibrate 5 times with 10 µl of 0.1% TFA. Bind the tryptic peptides by pipetting the sample up and down 15 times. Wash twice with 10 µl of 0.1% TFA.
4. Elute the purified tryptic peptide mixture with 6 µl of 85% v/v acetonitrile/0.1% v/v TFA by pipetting up and down for 10 times. The eluate is air-dried. Prior to MS analysis, redissolve the dried tryptic peptide mixture with 6 µl of 2% actonitrile/0.5% acetic acid.
5. The tandem mass spectrometry analysis of the purified tryptic peptide mixture is carried out with a capillary liquid chromatography (LC)- electrospray

ionization (ESI)-quadropole ion-trap (Q-IT) mass spectrometer system (LCQ^{Deca}; ThermoFinnigan, San Jose, CA).

6. Set the LCQ^{Deca} instrument parameters as follows: ESI voltage 2.0kV, capillary probe temperature 110°C, and electron multiplier −900V. Use a solution (1pmol/ul) of synthetic des-arg bradykinin to determine the instrument sensitivity and mass accuracy. In the MS mode, the $(M + 2H)^{2+}$ ion of des-arg bradykinin at *m/z* 452.7 should have a signal intensity of 1×10^7 (arbitrary units) at a flow-rate of 0.5µl/min. In the MS/MS mode, the precursor ion at *m/z* 452.7 should produce *m/z* 404.2, 710.4, and 807.4. Acquire the MS/MS data in the "triple-play" data-dependent scan mode that includes four scan events: a full-range MS scan, and three MS/MS scans that depend on the "triple-play" data scan mode.
7. Inject manually 6µl tryptic peptide mixture into the LC system. Separate the peptide mixture on a capillary column: New Objective PicoFrit 360µm (OD), 75µm (ID), 15µm tip pores (ID) packing an 8cm length Magic C18AQ material material (5µm beads, 200 Å pores) (Michrom Bioresources, Auburn, CA). Elute (35µl/min flowrate) peptides with the following five gradients (Solution A = water + 0.1% v/v formic acid; Solution B = 90% v/v acetonitrile + 0.1% v/v formic acid): (1) 100% solution A and 0% solution B (5min); (2) linear gradient up to 35% solution A and 65% solution B within 30min; (3) maintain 35% solution A and 65% solution B (15min); (4) linear gradient down to 100% solution A and 0% solution B within 5min; and (5) maintain 100% solution A and 0% solution B. The peptides elute directly into the ESI source. All MS/MS data are acquired and managed with the ThermoFinnigan Xcalibur software.
8. Identification of a nitrotyrosine-containing protein. Use amino acid sequence to identify the protein by searching the SWISS-PROT and NCBInr databases with the SEQUEST software that is a part of the LCQDeca software package. Mass modifications of +45kDa (+ NO_2–H) at Tyr, of +57kDa (+NH_2COCH_2–H) at Cys, and of +18kDa at Met are considered in the search. Each positive search result – nitration of a Tyr residue – is confirmed with a manual check of the original LC, MS, and MS/MS data to determine each nitration site. During the analysis of those nitrated proteins, the following experimental criteria are applied: K or R at the C-terminus; K, R, or D ***(1)*** preceding the N-terminus; 0 or 1 missed trypsin cleavage sites; singly charged product b- and y-ions, and Homo sapiens. *De novo* sequencing is used to independently and accurately determine each amino acid sequence. The amino acid sequence from *de novo* sequencing is used to search the human SWISS-PROT protein database with the SIB BLAST search engine (http://us.expasy.org/tools/blast/). An example **(Fig. 2)** demonstrates the identification of a nitrotyrosine-containing protein.

4. Notes

1. The water that is used to make all buffers/solutions and to wash gels should be deionized distilled (dd) water that has a resistivity of $18.2\,M\Omega^{-cm}$. We often use Millipore water.

2. Tetranitromethane is an oxidizing reagent; very toxic; irritating to the eyes, respiratory system, and skin with very serious irreversible effects. Experimental gloves should be worn, and one should operate in the hood.
3. Butylated hydroxytoluene (BHT) is an effective antioxidant to protect a sample from oxidation.
4. Acrylamide and bisacrylamide in the monomeric form are neurotoxins. Avoid inhaling and exposing to skin. Dispose ecologically of the remains by polymerizing the remains with an excess of ammonium persulfate ***(21,22)***.
5. Use fresh ammonium persulfate solution. Fresh ammonium persulfate "crackles" when water is added. It it does not, replace it with fresh stock.
6. High temperature will decompose urea. Work at < to 30°C.
7. For "protein extraction buffer" and "rehydration buffer", generally a stock solution (7 *M* urea, 2 *M* thiourea, 4% (w/v) CHAPS, and trace bromophenol blue) is made, and stored in 1 ml aliquots at −80°C. Prior to use, the protein extracting buffer is made by adding 15.4 mg DTT (final concentration = 100 m*M*) and 20 µl pharmalyte (final concentration = 0.5%) to 1 ml of rehydration stock solution, and mix well. The rehydration buffer is made by adding 9.2 mg DTT (final concentration = 60 mM) and 5 µl Pharmacia IPG buffer (final concentration = 0.5%) to 1 ml of rehydration stock solution, and mix well. Thus, DTT, pharmalyte, and IPG buffer will be fresh.
8. For "reducing equilibration buffer" and "alkylation equilibration buffer," generally an SDS equilibration stock buffer (375 m*M* Tris-HCl pH 8.8, 6 *M* urea, 2% (w/v) SDS, 20% (v/v) glycerol, and trace bromophenol blue) is made, and stored in aliquots of 50 ml at –80°C. Prior to use, the reducing equilibration buffer is made by adding 1 g DTT (final concentration = 2% w/v) to 50 ml of SDS equilibration stock buffer, and mixed well. The alkylation equilibration buffer is made by adding 1.25 g iodoacetamide (final concentration = 2.5% w/v) to 50 ml of SDS equibration stock buffer, and mix well. Thus, DTT and iodoacetamide will be fresh.
9. SDS is a strong detergent that irriates the respiratory system. Avoid inhaling the SDS powder when SDS is weigh.
10. The Agarose solution should not be boiled over. If the solution turns light yellow, it should be replaced with a fresh agarose solution.
11. There are commonly two type of Immobilon-P transfer membrane: 0.45 µm and 0.2 µm. The 0.45-µm PVDF membrane is often used to transfer the protein, whereas the 0.2-µm PVDF membrane is often used to transfer small molecular weight proteins or peptides. If a 0.2-µm PVDF membrane is used to transfer protein, then there will be more resistance that decreases the transfer efficiency.
12. The anti-nitrotyrosine antibody is currently a commercial product that can be obtained from Upstate Biotechnology, International Chemicomn, and

Sigma, etc. A polycolonal or monocolonal antinitrotyrosine antibody can be obtained.

13. Pierce Immobilized protein G plus cross-linked 6% beaded agarose supplied as a 50% slurry (for example, 400 µl of settled gel is equivalent to 200 µl of 50% slurry) that contains 0.02% sodium azide. Binding capacity is > 20 mg human IgG per ml of settled gel.
14. Disuccinimidyl suberate (DSS) is a very strong crosslinker, whose activity is much stronger than DMP, another commonly used crosslinker. DSS is water-insoluble and should be first dissolved in an organic solvent such as DMSO or DMF, and added to the aqueous reaction mixture. DSS is moisture-sensitive. Store at 4–8°C in a desiccator. To avoid moisture condensation onto the product, the vial that contains DSS must be equilibrated to room temperature before opening. Prepare DSS solution just prior to use because DSS is readily hydrolyzed and becomes non-reactive. Over-crosslinking proteins with biological activity (e.g., enzymes, antibodies, etc.) could result in loss of activity possibly because of a conformational changes of the molecule. Activity loss also may occur when DSS modifies lysine groups that are involved in binding substrate or antigen. Adjusting the molar ratios of reagent to the target may overcome activity loss. Alternatively, use a cross-linker that targets a different functional group. Hydrolysis of DSS is a competing reaction and increases with increasing pH. Hydrolysis occurs more readily in dilute protein or peptide solutions. In concentrated protein solutions, the acylation reaction is favored.
15. Promega's Sequencing Grade Modified Trypsin is porcine trypsin modified by reductive methylation. Its resistance to proteolytic digestion is two times greater activity than unmodified trypsin. Sequencing Grade modified trypsin is further treated with TPCK followed by affinity purification to yield a highly active and stable molecule. Modified trypsin has maximal activity at pH 7–9, and is reversibly inactivated at pH 4. It is resistant to mild denaturing conditions: 0.1% SDS, 1 *M* urea, or 10% acetonitrile. Modified trypsin retains 48% activity in 2 *M* guanidine HCl.
16. It is important to wash the blood from the tissue surface. Care should be taken with small tissue samples because of loss of tissue.
17. In order to obtain consistent results among different samples when comparing gels, a lyophyilized pituitary is redissolved in a solution of 8 *M* urea and 4% CHAPS for BCA protein concentration analysis because BCA reagent is not compatible with thiourea, DTT, IPG buffer, and bromophenol blue. For different experiment, each time use the protein concentration of a fixed sample to verify the protein concentration of other samples.
18. The standard curve is an inverted "S"-like curve. The sample points that correspond to the linear section of the inverted "S"-like curve are used to determine the nitrotyrosine concentration of sample.

19. PVDF membrane with protein can be kept in blocking solution for 2–3 days at 4°C with gently shaking.
20. After blocking, incubation with primary antibody, and incubation with secondary antibody, wash fully because salt and Tween-20 could result in particles on the visualized PVDF that would increase the background.
21. The size of the commercially Amersham immobilized pH gradient dry strip is 0.5-mm thick and 3-mm wide with different length (7, 11, 13, 18, and 24 cm). The pH range is 3–10, 4–7, 6–9, 4–5, etc., with a linear pH gradient or non-linear pH gradient. The pH range of the IPG buffer should be consistent with the pH range of the IPG strip; for example, IPG buffer pH 3–10 NL matches the IPG stip pH 3–10 NL, IPG buffer pH 3–10 matches the IPG strip pH 3–10 (22).
22. It is necessary for a rehydrated IPG strip to be rinsed fully with ddH_2O to remove excess mineral oil and undissolved components.
23. The 110-cm long electrode paper strips not only conduct but also remove the salt from samples. The latter significantly improves the IEF effect because tissue samples all contain salts that will affect the IEF. If the salt is too high, during the IEF, the electrode paper strips may be replaced once with a new electrode strip.
24. After IEF, use only water and liquinox detergent (hot water works nicely to remove mineral oil) to clean electrodes, Dry strip tray, and strip aligner. Use paper towel to clean the cooling plate.
25. The cast gels can be used within a week when they are covered with moist filter paper and stored at 4°C. Moreover, for our 2-D gel studies, the reproducibility of a single-concentration gel is better than a gradient gel ***(23,24)***.
26. If the gel plate is completely immersed in buffer, then there will be a significant current leak that negatively affects the electrophoresis results. If the buffer level is not high enough to cover the entire gel area, then the end of the gel will show no separation.
27. After electophoresis, use only water to wash the Dodeca tank, gaskets, electrode cards, and lid. For long-term storage, flush all parts thoroughly with water to completely remove any residual buffer.
28. A PVDF membrane has two different sides: hydrophobic and hydrophilic. The water moves slowly on the hydrophilic side, and fast on the hydrophobic side. The protein should be transferred to the hydrophilic side of PVDF.
29. A PVDF membrane must always be kept wet; if it dries, then re-wet in methanol and water.
30. More attention should be paid to avoid any air bubbles in **steps 1** to **7**) because they severely affect the semi-dry electrotransfer.
31. Our studies ***(23,24)*** have demonstrated the high levels of reproducibility and resolution of 2DGE. For the first dimension IEF, a high reproducibility

can be obtained with commercially available IPGstrips. For the second-dimensional SDS-PAGE, different types of gel system are available: vertical versus horizontal gel systems; constant-percentage versus gradient gels. Compared to the horizontal Multiphor gel system (pre-cast gradient SDS gel = 180 × 245 × 0.5 mm; analyzes a gel at a time), the vertical Dodeca gel system (single-concentration gel = 190 × 205 × 1 mm; analyze up to 12 gels at a time) demonstrates a better spatial and quantitative reproducibility ***(23)***, and a wider linear dynamic range to measure the change in the protein abundance in a complex proteome ***(24)***. Moreover, for between-gel comparison, the same experiment conditions (including the loading amount of protein, sample-dissolving solution, IEF, IPGstrip equilibration solution, SDS-PAGE, and gel image process) should be used to conduct two-dimensional gel electrophoresis experiments and image analysis for each experiment ***(25)***.

32. In these experiments, especially for digestion with trypsin, gloves and a head cap are always used to avoid any keratin from skin and hair contamination of the protein sample ***(20)***.
33. All tubes and pipette tips that contact protein sample or peptides should be siliconized (or low-retention) to avoid any loss of protein or peptide.

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Mass Spectrometric Determination of Protein Ubiquitination

**Carol E. Parker, Maria Warren Hines, Viorel Mocanu,
Susanna F. Greer, and Christoph H. Borchers**

1. Introduction

Cellular homeostasis requires a delicate balance of protein synthesis and degradation, a balance which is maintained by the actions of regulatory proteins. In turn, proteins which are no longer required must be degraded in a rapid and selective manner. The selective degradation of proteins involved in diverse regulatory mechanisms such as signal transduction ***(1)***, transcriptional regulation ***(2)***, cell cycle regulation ***(3)***, and stress response ***(4)*** have been linked to the covalent attachment of ubiquitin to proteins. Ubiquitin is a 76-residue polypeptide, which attaches via its carboxy-terminus to lysine ε amino groups of the target protein. Ubiquitin-protein conjugates are short lived, primarily because of proteolysis by the 26S proteosome or, in some cases, dissociation of the complex with removal of the ubiquitin by ubiquitin isopeptidases ***(5)***. Other roles for monoubiquitination have recently been uncovered, including acting as a signal for protein trafficking, cell division, targeting proteins to subnuclear structures, endocytosis, signal transduction, and kinase activation ***(6–11)***. Both the dynamic nature of these important regulatory proteins, and the low protein levels *in vivo,* make analysis of protein ubiquitination inherently difficult.

The use of mass spectrometry to identify sites of ubiquitination within target proteins *in vivo* will allow greater understanding of the ways in which ubiquitination alters protein function. Therefore, the development of broadly applicable identification approaches is critical. The importance of ubiquitination and the role of mass spectrometry in the study of ubiquitination has recently been reviewed by Kirkpatrick, Denison, and Gygi ***(12,13)***, Xu and Peng ***(14)***, and Drews, Zong, and Ping ***(15)***.

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2. Materials

2.1. MALDI Analysis of Intact Proteins

1. Bruker Reflex III or a Bruker Ultraflex matrix-assisted laser desorption (MALDI-MS, (Bruker Daltonics, Billerica, MA).
2. α-cyano 2-hydroxycinnamic acid (Aldrich; St. Louis, MO).
3. Ethanol.
4. Water, HPLC-grade.
5. Formic acid.

2.2. LC/MS/MS

1. Waters/Micromass Q-TOF (Waters/Micromass Corp., Milford, MA).
2. PepMap C_{18} 15 cm × 75 µm id capillary column (Dionex; Sunnyvale, CA).
3. Trapping column 5 mm × 800 Å id C_{18} P3 (Dionex).
4. Water, HPLC-grade (Pierce).
5. Acetonitrile, HPLC-grade (Pierce).
6. Formic acid.

2.3. Affinity Purification

1. Anti-HA antibody beads (Sigma, St. Louis, MO).
2. Anti-FLAG antibody beads (Sigma).
3. Ammonium bicarbonate.
4. Ethanol.
5. Water (Pierce).
6. Formic acid (Fisher).

2.4. In-Solution Digestion

1. Water, deionized or HPLC-grade (Pierce).
2. Trypsin, sequencing-grade (Promega; Madison, WI).
3. GluC (Sigma).
4. Ammonium bicarbonate.
5. Low-retention Eppendorf tubes (Axygen; Union City, CA).
6. Thermomixer (Eppendorf; Hamburg, Germany).

2.5. Lyophilization and Reconstitution

1. Freeze dryer (Labconco; Kansas City, MO).
2. Water (Pierce).

3. Methods

3.1. Evidence from Gels.

Most direct evidence for ubiquitination comes from gel electrophoresis (**Fig. 1**), where a series of higher molecular weight bands are observed above the molecular weight of the protein, or from Western blot analysis using anti-ubiquitin antibodies **(Fig. 2)**.

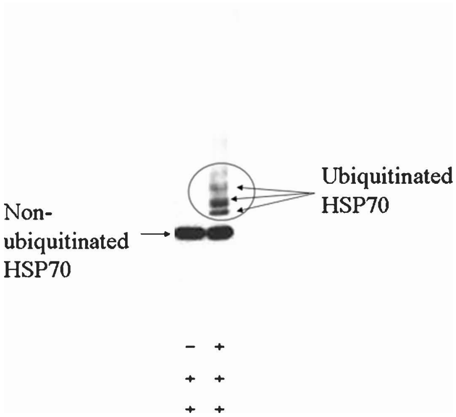


Fig. 1. Ubiquitination evidence from Gels. PAGE gel showing a series of bands above the molecular weight of the non-ubiquitinated protein. (Collaborator: W. C. Patterson).

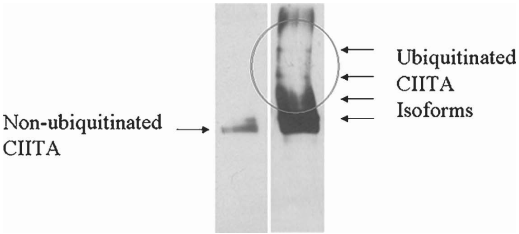


Fig. 2. Ubiquitination evidence from Western blot analyses. PAGE gel showing a series of bands above the molecular weight of the non-ubiquitinated protein: Western blot analysis of CIITA, showing ubiquitination.

3.2. Mass Spectrometric Evidence for Protein Ubiquitination

While ubiquitination is clearly important for protein degradation, most mass spectrometric studies on ubiquitination have focused on protein phosphorylation, rather than direct mass spectrometric studies of protein ubiquitination. There are three types of mass spectrometric evidence for ubiquitination: the first is ubiquitination of the intact protein, the second is co-electrophoretic migration of the target protein and the attached ubiquitin and, and the third is the mass shift of a ubiquitinated peptide relative to the nonubiquitinated peptide.

3.2.1. Evidence from MS of the Intact Protein

Direct mass spectrometric evidence of intact ubiquitinated HSP70 is shown in **Fig. 3**. This spectrum was obtained by direct MALDI-MS analysis of ubiquitinated HSP70, affinity-bound to anti-HSP70 beads.

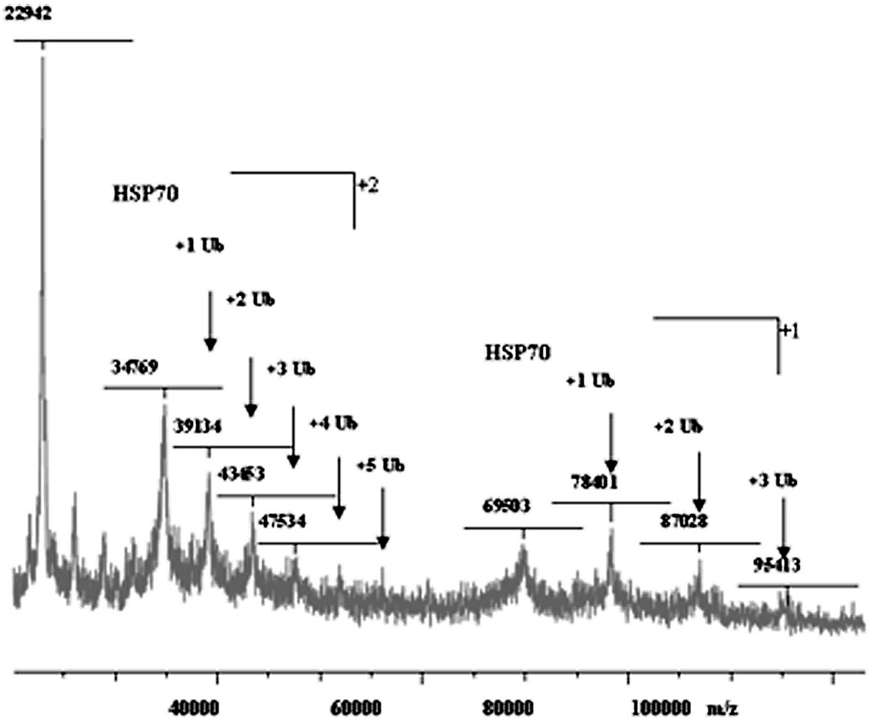


Fig. 3. Ubiquitination evidence from MALDI of intact proteins. MALDI-MS of HSP70 with a series of ubiquitin attached moieties.

3.2.1.1. Direct MALDI Analysis of Proteins Bound to Affinity Beads (*see* Note 1)

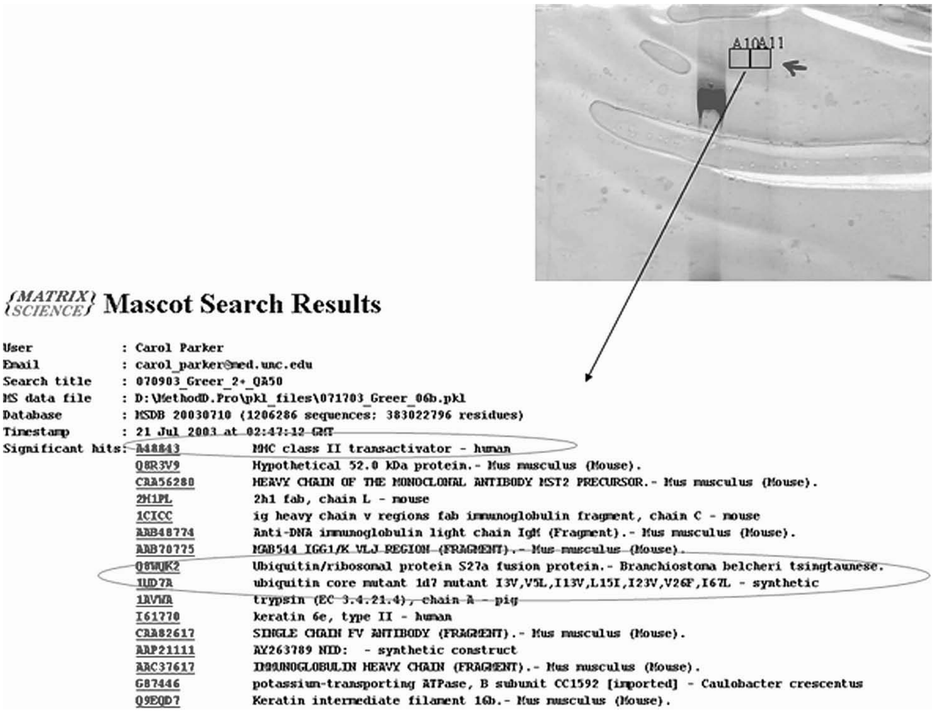
1. Use antibodies *covalently* bound to the affinity beads (*see* **Note 2**).
2. Bind the antibody according to the bead manufacturers' instructions (*see* **Notes 3** and **4**).
3. Rinse beads 3 times with 2–3 bead volumes of 100 mM ammonium bicarbonate (*see* **Note 5**).

3.2.1.2. Spotting the MALDI Target

1. Prepare the MALDI matrix solution – a saturated solution of recrystallized (*see* **Note 6**) α-cyano 2-hydroxycinnamic acid in 45:45:10 ethanol:water:formic acid. (*see* **Note 7**).
2. Pipet 0.5 μL of settled beads onto the MALDI target, followed by 0.5 μL of MALDI matrix solution.

3.2.2. Evidence from MS and MS/MS of Peptides

The second type of mass spectrometric evidence for ubiquitination comes from in-gel digestion and protein identification studies. In-gel digestion of the higher molecular weight bands followed by protein identification by MALDI-MS



{MATRIX SCIENCE} Mascot Search Results

```
User            : Carol Parker
Email           : carol_parker@med.unc.edu
Search title    : 070903_Greer_2+_QA50
MS data file    : D:\MethodD.Pro\pkl_files\071703_Greer_06b.pkl
Database        : MSDB 20030710 (1206286 sequences; 383022796 residues)
Timestamp       : 21 Jul 2003 at 02:47:12 GMT
Significant hits: A48843         MHC class II transactivator - human
                  Q8R3V9         Hypothetical 52.0 kDa protein.- Mus musculus (Mouse).
                  CAA56280       HEAVY CHAIN OF THE MONOCLONAL ANTIBODY MST2 PRECURSOR.- Mus musculus (Mouse).
                  2H1PL          2h1 fab, chain L - mouse
                  1CICC          ig heavy chain v regions fab immunoglobulin fragment, chain C - mouse
                  AAB48774       Anti-DNA immunoglobulin light chain IgM (Fragment).- Mus musculus (Mouse).
                  AAB70775       MAB544 IGG1/K VLJ REGION (FRAGMENT).- Mus musculus (Mouse).
                  Q8WUK2         Ubiquitin/ribosomal protein S27a fusion protein.- Branchiostoma belcheri tsingtaunese.
                  1UD7A          ubiquitin core mutant 1d7 mutant I3V,V5L,I13V,L15I,I23V,V26F,I67L - synthetic
                  1AVWA          trypsin (EC 3.4.21.4), chain A - pig
                  I61770         keratin 6e, type II - human
                  CAA82617       SINGLE CHAIN FV ANTIBODY (FRAGMENT).- Mus musculus (Mouse).
                  AAP21111       AY263789 NID:  - synthetic construct
                  AAC37617       IMMUNOGLOBULIN HEAVY CHAIN (FRAGMENT).- Mus musculus (Mouse).
                  G87446         potassium-transporting ATPase, B subunit CC1592 [imported] - Caulobacter crescentus
                  Q9EQD7         Keratin intermediate filament 16b.- Mus musculus (Mouse).
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Fig. 4. Peptides from the target protein CIITA and peptides from ubiquitin from a gel band at mw ~200 KDa.

or LC/MS/MS on a gel can sometimes provide evidence for ubiquitination – evidence comes from peptides rather than proteins. In the example shown in **Fig. 4**, peptides from the target protein CIITA and peptides from ubiquitin were found in a gel band of approximately 200 Kda, clearly higher than the unmodified 150 kDa CIITA or the 8.7 kDa unbound modified monoubiquitin (*see* **Subheading 3.1.2.4**).

3.2.3. MS and MS/MS Spectra of Ubiquitinated Peptides

The third type of mass spectrometric evidence comes from the modified peptide itself, either from a shift in peptide molecular weight, or from MS/MS data. Ubiquitin is a 76-amino acid protein. An E3 ligase attaches ubiquitin to the ε amino group of a lysine residue in the target protein. This covalent linkage is formed at C-terminal glycine residue of the ubiquitin, with loss of the elements of water. Cleavage with trypsin or gluC (*see* **Note 8**) leaves characteristic “tails” on the modified lysine. These “tails” cause a shift in the molecular weight of the peptide, which can be used to distinguish these modified peptides from the unmodified peptides (**Fig. 5**). The actual site of ubiquitination can then be determined by MS/MS sequencing.

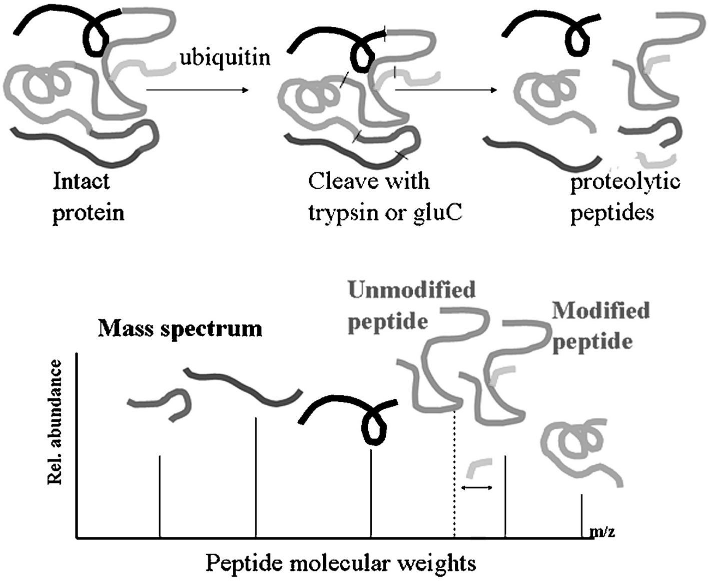


Fig. 5. Schematic showing the expected peptide molecular weight shift caused by ubiquitination.

These peptide mass shifts can be used to find and sequence ubiquitinated peptides. Mascot, for example, allows the user to create a modification of a particular mass, which can then be used to search the peptide data from a given digest. Due to their transient nature and low natural abundances, ubiquitinated peptides are difficult to detect. A study by Gururaja et al ***(16)*** on Hela cell lysates, used a 6 × his-tagged ubiquitin, IMAC purification, digested by Lys-C and trypsin, and protein identification by 2D-LC/MS/MS using strong cation exchange and C18 reversed phase media. Ubiquitination was confirmed by anti-his tag and anti-ubiquitin Western blotting on the undigested lysates. A total of 244 proteins were found, which the authors categorized into functional groups, but determination of the exact ubiquitination sites was not the focus of this study.

Very few ubiquitinated peptides were found by mass spectrometry from a single preparation until the work by the Gygi group in 2003 ***(17)***. As in the studies described above, a 6 × his-tagged ubiquitin was also used, and ubiquitinated proteins from yeast were purified by IMAC. The ubiquitin-enriched fraction (0.2 mg out of each original 100 mg of yeast cell lysate) was then digested with trypsin, fractionated into 80 fractions on a SCX column, and the 80 fractions were analyzed by capillary LC/MS/MS. Peptide MS/MS data was searched using Sequest software, allowing for a ubiquitin-modified lysine with a mass shift of +114 Da. In this manner, 110 ubiquitination sites were determined. In another study using 6 × his-myc-Ub, also from the Gygi group, 211 proteins were identified. Ubiquitination sites were identified on 15 of these proteins ***(18)***. A detailed description of this method has been reported by Peng and Cheng ***(19)***.

Using a similar approach, Kirkpatrick et al ***(20)*** also studied the "ubiquitinome" of human cells, this time HEK293 cells. As in the above studies, 6 × his-tagged ubiquitin was used, followed by Ni-IMAC purification. Lysates from as many as 80 plates were pooled, and the eluted proteins were digested with trypsin, followed by LC/MS/MS. Twenty-two ubiquitinated proteins were identified, along with 19 additional proteins non-specifically-bound to the IMAC beads. An attempt was made to find the ubiquitinated peptides, but only branched peptides from polyubiquitin were identified in this study – by the presence of the GG modification on lysine. No consistent fragmentation (i.e., no loss of 114 Da) was observed in these GGK-modified peptides. Confirmatory evidence of the ubiquitination at these specific sites was the lack of tryptic cleavage at the modified K.

An alternative, but still "shotgun" approach is to use an anti-ubiquitin antibody to accomplish the enrichment. This approach was used by Figeys' group, and the digestion of 30 bands resulted in the identification of 70 proteins ***(21)***. Several GGK-containing "signature" peptides were located by their mass shifts, using a Mascot database search. An interesting and useful improvement on the anti-ubiquitin affinity purification approach was the use of the anti-*poly*ubiquitin antibodies which had been developed in 1994 by Fujimuro et al ***(22).*** The use of one of these antibodies (FK2) instead of a conventional anti-ubiquitin antibody has been shown to work even in denaturing conditions and improves the selectivity of ubiquitinated protein enrichment by avoiding the capture of free ubiquitin ***(23,24)***.

3.2.3.1. MS/MS Fragmentation of Ubiquitinated Peptides

When the work described in this book chapter was initiated (2004), we could only find two reports of ubiquitination site determination which showed detailed MS/MS spectra of ubiquitinated peptides. These were on specifically-targeted proteins of interest – the first was the work of Laub et al ***(25)***, who used gluC for proteolytic digestion of the protein, and the second was the work of Dohlman's group ***(26)***, who used trypsin. Both of these papers showed MS/MS spectra of the ubiquitinated peptides, and we decided to use these peptides as the starting point for a detailed study of the fragmentation of ubiquitinated peptides with the goal of finding specific diagnostic fragment ions to aid in detecting low levels of ubiquitinated peptides at lower levels in biological materials ***(27)***.

When a ubiquitinated protein is enzymatically digested, a portion of the ubiquitin side chain remains attached to the modified lysine **(Fig. 6)**. A ubiquitinated peptide therefore has 2 N-termini – one from the original peptide and one from the ubiquitin side chain. Thus, it is possible to have two series of b ions and y

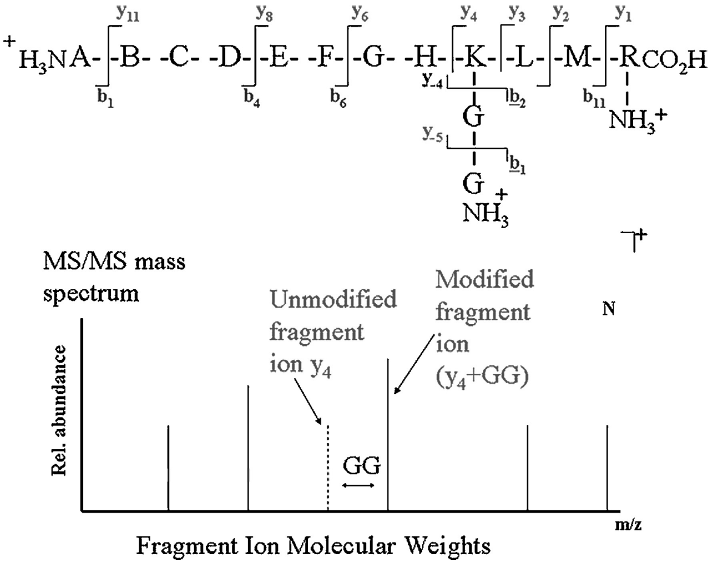

+H3NA- -B- -C- -D- -E- -F- -G- -H- -K- -L- -M- -RCO2H
y11
y8
y6
y4
y3
y2
y1
b1
b4
b6
y-4
b2
b11
NH3+
G
y-5
b1
G
NH3+
MS/MS mass spectrum
Unmodified fragment ion y4
Modified fragment ion (y4+GG)
GG
N
Rel. abundance
m/z
Fragment Ion Molecular Weights

Fig. 6. Schematic showing fragmentation of ubiquitinated peptides.

ions. For the sake of clarity, we have chosen to refer to those *b* and *y* ions involving the ubiquitin side chain as *b* and *y* ions. Obviously, diagnostic ions for the modification must come from fragmentation of this side chain. Fragment ions involving any part of the "normal" peptide will vary in mass according to the peptide being modified and will therefore not be of general diagnostic use.

3.2.3.2. Examination of the Literature Tryptic Peptide Spectra

MS/MS spectra of GG-tagged tryptic peptides are shown in both the paper from Dohmann's group, and that of the Gygi group. Examination of these spectra show that *b* and *y* ions are produced by dissociation of the peptide from the target protein, but there do not appear to be any *b* ions from the GG side chain, or the GGK portion of the peptide.

3.2.3.3. Examination of the Literature gluC Peptide Spectra

The MS/MS spectrum of the gluC ubiquitinated peptide from rXL-calmodulin which was shown in the Laub et al paper *(25)*, reveals b and y ions from the calmodulin portion of the peptide **(Fig. 7)**. Interestingly, it also shows doubly-charged (b$_7$-H$_2$O) $^{2+}$, (b$_{14}$-H$_2$O)$^{2+}$, and (b$_{12}$-H$_2$O)$^{2+}$ ions which contain both of the N-termini (one from the calmodulin peptide and one from the ubiquitin side chain) of the original branched peptide. These two ions (athough of low relative abundance) can be seen in the MS/MS spectrum of the ubiquitinated peptide from natural BT-calmodulin. Careful examination of the both spectra

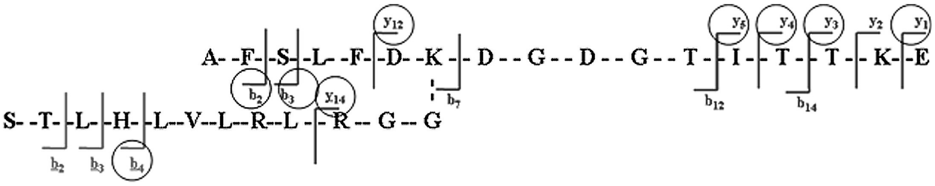

A - F - S - L - F - D - K - D - G - D - G - T - I - T - T - K - E
S - T - L - H - L - V - L - R - L - R - G - G
y12
y14
y5
y4
y3
y2
y1
b2
b3
b7
b12
b14
b2
b3
b4

Fig. 7. Calmodulin ubiquitinated peptide structure after cleavage with gluC. Circled peptide fragments were not noted in the original publication by Laub et al *(25)*, but appear to have the appropriate masses.

reveals ions which might be from the ubiquitin side chain, and therefore of possible diagnostic utility. The y_{14} ion includes ions from both the side chain and the C-terminus of the calmodulin-portion of the peptide, so it cannot be used as a ubiquitination marker ion. However, there are also ions from the side chain, b_4 at m/z 478.4 (obs) and, possibly, b_2, at m/z ~191.2 (obs), which appear to be diagnostic ions of ubiquitination, after digestion with gluC.

3.2.3.4. PREPARATION OF MODEL UBIQUITINATED PEPTIDES

In order to further study the fragmentation of ubiquitinated peptides, we had a model peptide synthesized. A peptide was synthesized with the structure shown in **Fig. 7**. These modified peptides should show a characteristic mass shift from their unmodified analogues: in the case of trypsin, a mass shift of 114.0428 Da from the GG "tag" left on the modified lysine. In the case of gluC, a mass shift of 1302.7883 Da, a much larger mass shift resulting from the much longer "tail" (STLHLVLRLRGG-) on the modified lysine, is expected. Unfortunately, the model peptide was synthesized with an amide at the C-terminus, and an acetyl group at the N-terminus, so it is not an exact model of the peptide found by Laub et al *(25)*. Fortunately, the acetyl group was in the "calmodulin" portion of the peptide, so we expected that diagnostic fragments from the ubiquitin side chain would still be present in the MS/MS spectrum.

ESI-MS/MS spectra of the model peptides were obtained by LC/MS/MS analysis using a Waters/Micromass Q-tof API US, equipped with a Waters capLC system. An aliquot of the sample was injected first onto a Dionex trapping column, which was then switched so that it was connected on-line to a 75 µm Dionex Pep-map analytical column and the ESI source. The original synthetic peptide, the model gluC peptide, was dissolved in water, and injected without further purification. It proved to be a mixture of acetylated and methylated forms, whose MS/MS spectra could be analyzed separately after separation by LC/MS/MS. The actual methylation sites can be deduced from the MS/MS spectrum: one is on the S in the calmodulin part of the peptide, the other is on one of the first two residues (S or T) of the ubiquitin tail.

The model ubiquitinated peptide was prepared from the synthetic peptide by means of an in-solution tryptic digest (see **Subheading 3.1.2.5.3**), using 2 µg of trypsin and 1 µg of the original peptide in 100 µL of 100 mM ammonium bicarbonate. As stated earlier, an aliquot of the digest was injected into the LC/MS/MS system, and went first onto a Dionex trapping column, which was then switched so that the column was connected on-line to a 75 µm i.d. Dionex Pepmap analytical column and the ESI source.

Two main products were formed: a peptide with the expected "GG" tail on the ubiquitinated lysine, and a second peptide with an "LRGG" tail, resulting from a missed cleavage. Even though this was an in-solution tryptic digest with a large amount of trypsin, a significant amount of a peptide was produced with a missed cleavage site on the side chain. Although this missed cleavage was unexpected, it is not unreasonable, since this cleave site is close to the branch point so cleavage at this site is likely to be sterically hindered. MS/MS spectra were obtained for both of these products. The formation of this peptide is of significant analytical interest as it provides a second characteristic molecular weight shift for ubiquitinated peptides after tryptic digestion.

3.2.3.5. Fragmentation of Model gluC Ubiquitinated Peptide

Because of the various possible dimethylated isoforms, the first model gluC peptide examined was the dimethylated version. The resulting spectrum is shown in **Fig. 8**. Both *b* and *y* fragment ions are found from the "normal" part of the peptide. Most interestingly, several fragments are found which only involve the side chain. As were observed in the literature spectrum, three characteristic ions were detected from the "ubiquitin" side chain: these are b_2, b_3, and b_4. These ions, in their un-methylated forms, (m/z 189.088, 302.172, 439.231, 555.315, and 651.383) should thus be diagnostic ions for peptides with ubiquitin side chains that have been cleaved with gluC.

3.2.3.6. Fragmentation of Model Tryptic Peptides

Cleavage of the synthetic model peptide with trypsin resulted in a GG-tagged peptide whose MS/MS spectrum is shown in **Fig. 9**. Unfortunately, as in the literature spectra, no characteristic fragment ions could be found which were diagnostic of the critical GGK portion of the molecule.

The MS/MS spectrum of the model tryptic peptide which had the LRGG tag (resulting from a missed cleavage) was more analytically useful **(Fig. 10)**. Diagnostic ions of the ubiquitin tag were found. These include b_2, b_4, and the internal fragment ion (LRGGK-28). There is also a LRGGKD ion fragment ion, but since this includes the "D" from the calmodulin peptide, it cannot be used as a diagnostic ion for modification by ubiquitin. The assignment of these peptide fragments is confirmed by the MS/MS spectrum of the S-methylated calmodulin

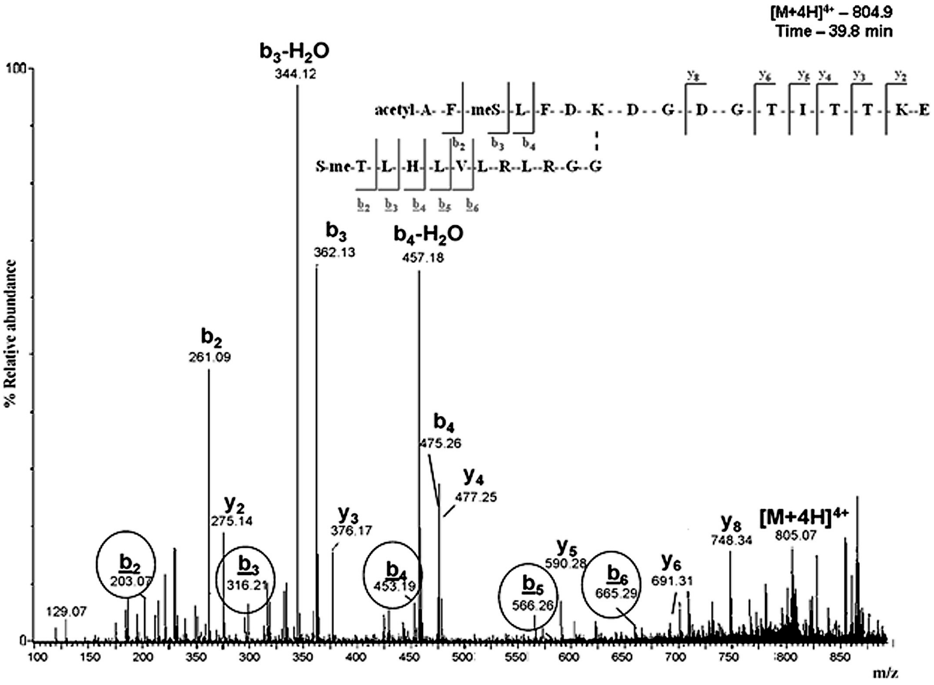


Fig. 8. MS/MS Fragmentation of synthetic model calmodulin gluC peptide, dimethyl form. (Reproduced with permission from ***ref. 27,*** copyright John Wiley & Sons, Limited).

peptide is shown in **Fig. 11**. The same diagnostic ions b_2, b_4, (LRGGK-28) ions are observed in this spectrum.

3.2.4. Enrichment of Samples for Specific Ubiquitin-Modified Proteins and/or Proteins That Interact with Them

As described above (**Subheading 3.2.3**), the work of Gururaja ***(16)***, Kirkpatrick ***(20)***, and Peng ***(17)*** depended upon enriching the sample in ubiquitinated peptides. Their goals were to find as many ubiquitination sites as possible in *any* protein, so their approach was to use a 6 × his-tagged ubiquitin, and enrich the sample in ubiquitinated proteins through the use of IMAC or 6 × his –myc-Ub ***(18)***. Other researchers have used antiubiquitin ***(21)*** or anti-polyubiquitin ***(23,24)*** for “shotgun” enrichment.

To find ubiquitination sites in a *particular* target protein requires a different approach. One method is the use of a GST-tagged substrate, and anti-GSH affinity beads. This method was used by the Marshall group for an FT-MS study of polyubiquitinated GST-Ubc5, ubiquitinated in vitro ***(28)***. FT-MS identified fifteen ubiquitination sites in GST-Ubc5, and four sites in ubiquitin, although large quantities of material were used (e.g., 700 μg GST-Ubc5).

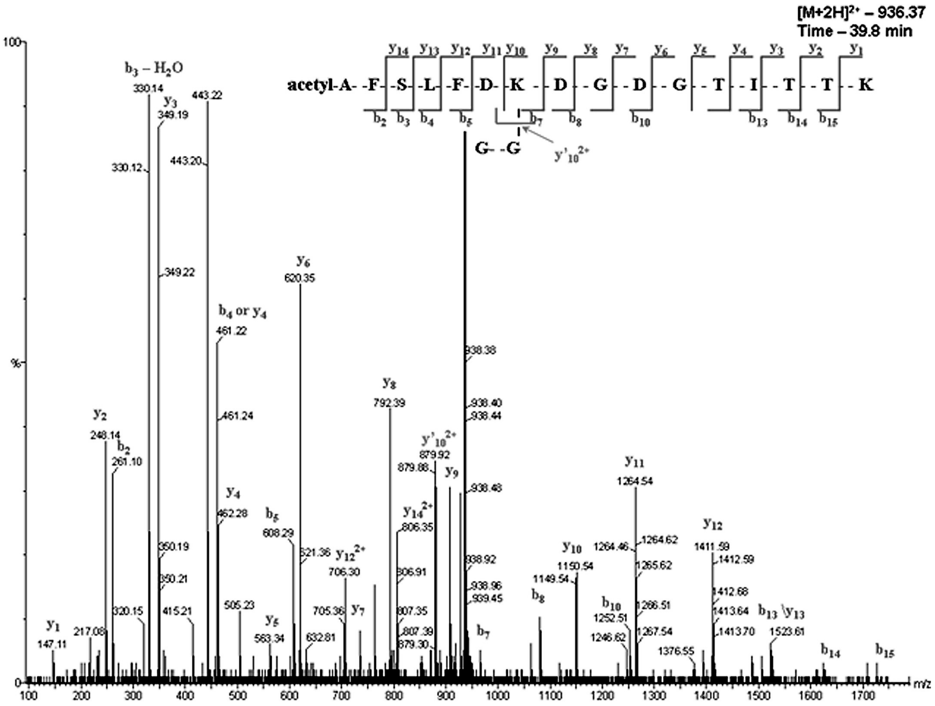


Fig. 9. Fragmentation of model "GG-" ubiquitinated tryptic peptide. Reproduced with permission from ***ref. 27,*** copyright John Wiley & Sons Limited.

If an antibody is available against the target protein, then it can be used to affinity purify the target protein from the cell lysate. If an antibody against the protein of interest is *not* available, an affinity tag can be incorporated into the target protein sequence. In our case, we used a FLAG tag added to the amino-terminus of the CIITA target protein so that it could be immunoprecipitated with anti-FLAG antibody beads.

As a second strategy for increasing the proportion of ubiquinated vs. non-ubiquitinated protein, a plasmid was used which coded for a modified ubiquitin which had all of the lysines modified to arginines and a HA tag on the C-terminus. This construct was designed to prevent the formation of polyubiquitin chains and thus to inhibit degradation of the target protein ***(29)***. The HA tag allows a second affinity purification step, either before or after proteolysis, this time on anti-HA beads. To avoid proteolysis of the ubiquitin (and loss of the HA tag), LysC was used for the initial digestion of the protein, instead of trypsin.

The affinity-bound protein can then be digested overnight with trypsin or with gluC (*see* **Note 8**) while still attached to the beads, using the protocols described in the next subheading. Alternatively, the protein can be eluted from the affinity beads with 1:1:8 ethanol:formic acid:water, lyophilized and digested in solution.

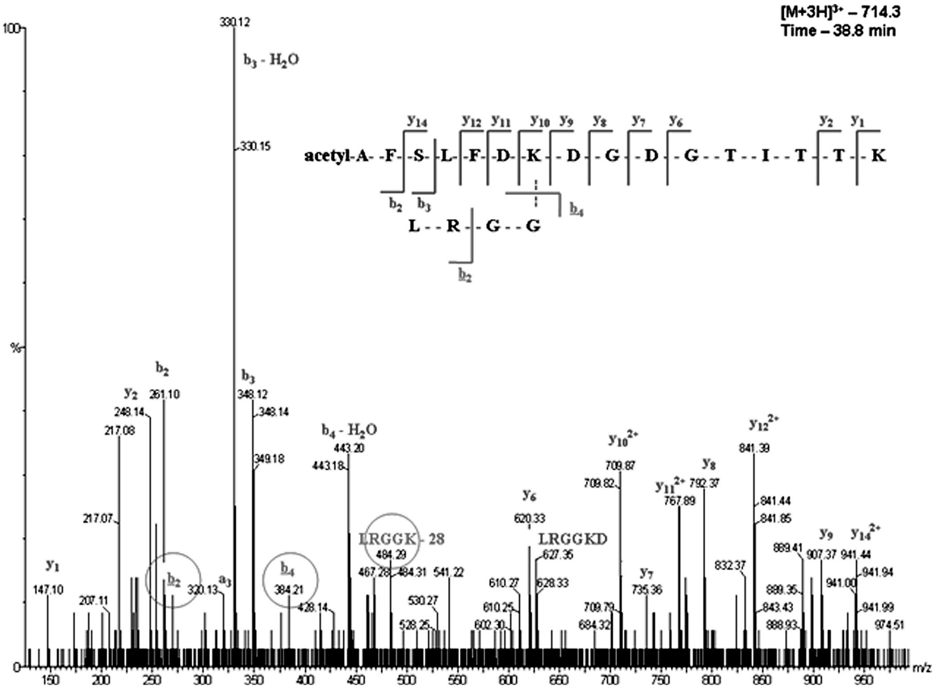


Fig. 10. Fragmentation of model "LRGG-" ubiquitinated tryptic peptide. (Reproduced with permission from ***ref. 27,*** copyright John Wiley & Sons Limited).

3.2.5. Elution and Enzymatic Digestion Procedures

3.2.5.1. ELUTION OF PROTEINS FROM BEADS

1. Place ~50–100 μL of beads in an Eppendorf tube.
2. Wash beads 3 × with 200 μL of 100 m*M* ammonium bicarbonate, and discard wash solutions.
3. Add 100 μL 1:1:8 Ethanol:formic acid:water.
4. Vortex, let settle.
5. Remove and save eluates.
6. Repeat extraction 2 more times.
7. Save and combine eluates.
8. Lyophilize.
9. Store at –80°C.

3.2.5.2. ON-BEAD DIGESTION TRYPTIC PROCEDURE (30) (SEE **NOTE 9**)

1. Place ~50–100 μL of antibody beads (with the attached affinity-bound protein) in an Eppendorf tube.
2. Wash 3 × with 200 μL of 100 m*M* ammonium bicarbonate.
3. Add 100 μL of 100 m*M* ammonium bicarbonate.

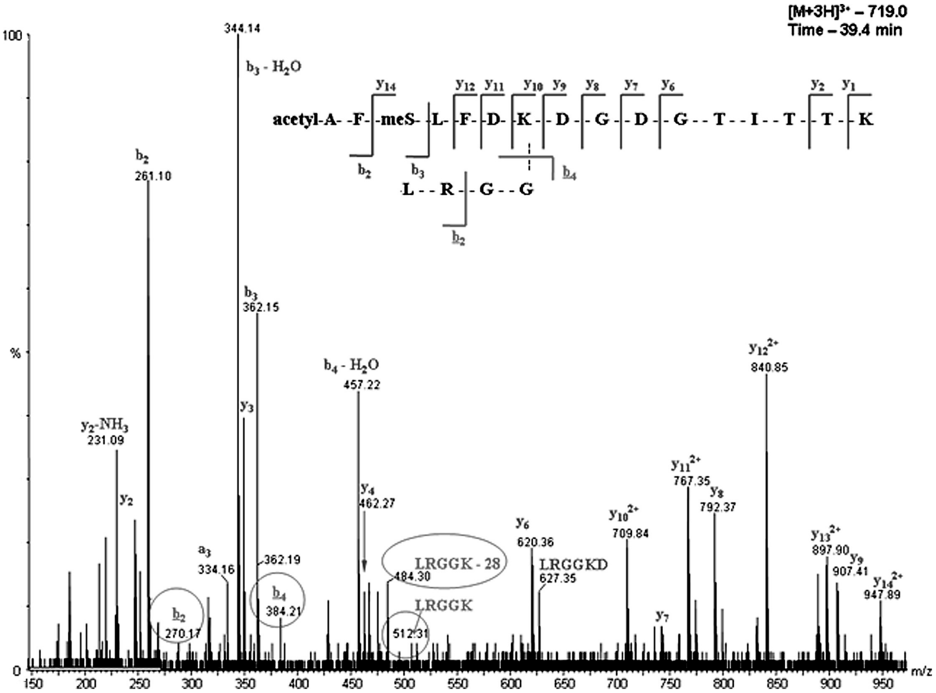


Fig. 11. Fragmentation of model "LRGG-" ubiquitinated tryptic peptide, methylated form. (Reproduced with permission from ***ref. 27,*** copyright John Wiley & Sons Limited).

4. Reconstitute Promega trypsin in 20 µL Promega resuspension buffer (0.015 *M* acetic acid) (Promega trypsin comes in aliquots of 20 µg per vial).
5. Add 2 µL trypsin solution to each sample (*see* **Note 10**).
6. Vortex/spin down at <1000 rpm to avoid breaking the beads.
7. Incubate overnight in sealed Eppendorf tubes, at 35°C with rotation (~400 rpm) (*see* **Note 11**).
8. Remove supernatant from beads.
9. Lyophilize to reduce volume (if sample is concentrated enough, supernatant can be injected directly).
10. Store at –80°C.

3.2.5.3. IN-SOLUTION TRYPTIC DIGESTION PROCEDURE

1. Calculate what a 1:50 Enzyme:Substrate ratio would be (*see* **Note 12**).
2. Reconstitute Promega trypsin in 20 µL Promega resuspension buffer (0.015 *M* acetic acid) (Promega trypsin comes in aliquots of 20 µg per vial.)
3. Dissolve sample in ~20 µL of 100 m*M* ammonium bicarbonate solution (*see* **Note 13**).
4. Add calculated amount of trypsin solution to each sample.
5. Vortex/spin down.

6. Incubate for at least 4 hrs for a digest in solution, or overnight for beads, in sealed Eppendorf tubes, at 35°C with rotation (~400 rpm).

3.2.5.4. In-Solution Digestion with gluC (*see* **Note 14**)

1. Dissolve the enzyme (which comes lyophilized, 25 µg per ampule) in 25 µL sample in water.
2. Dissolve sample in 100 µL of 100 m*M* ammonium bicarbonate buffer.
3. Add 2 µL enzyme solution.
4. Incubate overnight at 35°C, at 35°C with rotation (~400 rpm).
5. Freeze at −80°C.

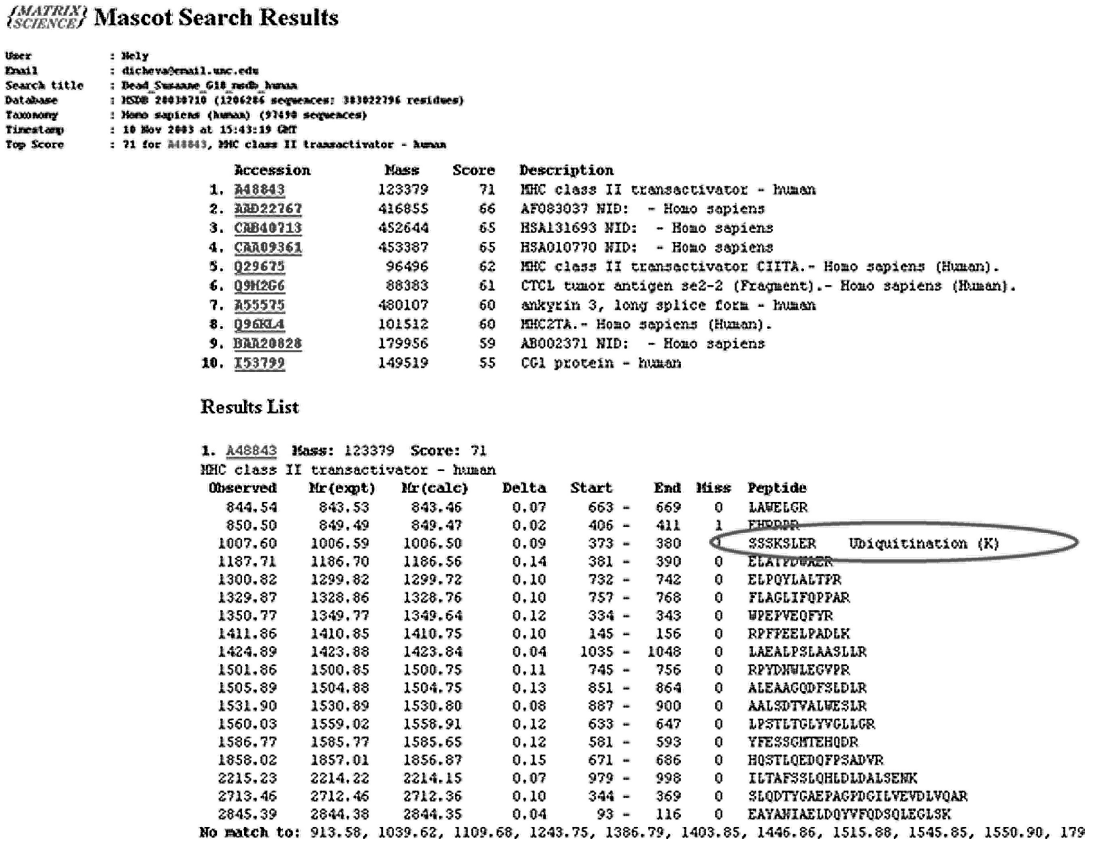
3.2.5.5. On-Bead Digestion with gluC (*see* **Note 14**)

1. Dissolve the enzyme (which comes lyophilized, 25 µg per ampule) in 25 µL sample in water.
2. Rinse ~50 µL of affinity beads 3 × with 100 µL of 100 m*M* ammonium bicarbonate buffer.
3. Add 100 µL of 100 m*M* ammonium bicarbonate to the beads.
3. Add 2 µL enzyme solution to the beads.
4. Incubate overnight at 35°C, at 35°C with rotation (~400 rpm).
5. Remove supernatant, and freeze at −80°C.

3.2.5. Mass Spectrometric Approaches for Detection of Ubiquitinated Peptides and Determination of Ubiquitination Sites

The first step is to simply perform LC/MS/MS on the peptide digest. Long runs with long linear gradients (>200 min) are preferred for separating complex mixtures of peptides in order to reduce suppression effects and to reduce the number of coeluting peptides, since selection of the precursor ions is done based on ion abundances. For an unknown modification site in a protein containing many potential sites, where the peptide molecular weight is therefore not known, automatic data-dependent triggering of MS/MS data collection (called "survey scan" mode in the Micromass MassLynx software) is the only feasible automatic scanning option.

The resulting MS/MS spectra are then analyzed by commercially-available software packages (such as Mascot or SEQUEST) which can be programmed (*see* **Notes 15** and **16**) to consider a lysine modified with a GG or, as we have learned from the above experiments on our model peptides, LRGG, for a tryptic digest, or with STLHLVLRLRGG for a gluC digest). Ideally, the ubiquitination will be found from this automated search routine (*see* **Notes 17** and **18**). **Fig. 12** shows an example of a database search of MALDI-MS data from a tryptic digest of a ubiquitinated protein. Here, a ubiquitinated peptide has been identified. Although promising, these results simply mean that there is a peak in the mass spectrum which has the mass of an expected tryptic peptide where a lysine has been modified with a GG tag. Since a peak at this mass could



```
{MATRIX}
{SCIENCE} Mascot Search Results

User          : Nely
Email         : dicheva@email.unc.edu
Search title  : Dead_Susanne_G18_msdb_human
Database      : MSDB 20030710 (1206286 sequences; 383022796 residues)
Taxonomy      : Homo sapiens (human) (97490 sequences)
Timestamp     : 10 Nov 2003 at 15:43:19 GMT
Top Score     : 71 for A48843, MHC class II transactivator - human

        Accession        Mass    Score   Description
    1.  A48843          123379     71    MHC class II transactivator - human
    2.  AAD22767        416855     66    AF083037 NID:  - Homo sapiens
    3.  CAB40713        452644     65    HSA131693 NID:  - Homo sapiens
    4.  CAA09361        453387     65    HSA010770 NID:  - Homo sapiens
    5.  Q29675           96496     62    MHC class II transactivator CIITA.- Homo sapiens (Human).
    6.  Q9H2G6           88383     61    CTCL tumor antigen se2-2 (Fragment).- Homo sapiens (Human).
    7.  A55575          480107     60    ankyrin 3, long splice form - human
    8.  Q96KL4          101512     60    MHC2TA.- Homo sapiens (Human).
    9.  BAA20828        179956     59    AB002371 NID:  - Homo sapiens
   10.  I53799          149519     55    CG1 protein - human

Results List

1. A48843  Mass: 123379  Score: 71
MHC class II transactivator - human
 Observed    Mr(expt)   Mr(calc)    Delta    Start    End  Miss  Peptide
   844.54     843.53     843.46     0.07     663 -    669    0   LAWELGR
   850.50     849.49     849.47     0.02     406 -    411    1   FHRPDR
  1007.60    1006.59    1006.50     0.09     373 -    380    1   SSSKSLER    Ubiquitination (K)
  1187.71    1186.70    1186.56     0.14     381 -    390    0   ELATPDWAER
  1300.82    1299.82    1299.72     0.10     732 -    742    0   ELPQYLALTPR
  1329.87    1328.86    1328.76     0.10     757 -    768    0   FLAGLIFQPPAR
  1350.77    1349.77    1349.64     0.12     334 -    343    0   WPEPVEQFYR
  1411.86    1410.85    1410.75     0.10     145 -    156    0   RPFPEELPADLK
  1424.89    1423.88    1423.84     0.04    1035 -   1048    0   LAEALPSLAASLLR
  1501.86    1500.85    1500.75     0.11     745 -    756    0   RPYDHWLEGVPR
  1505.89    1504.88    1504.75     0.13     851 -    864    0   ALEAAGQDFSLDLR
  1531.90    1530.89    1530.80     0.08     887 -    900    0   AALSDTVALWESLR
  1560.03    1559.02    1558.91     0.12     633 -    647    0   LPSTLTGLYVGLLGR
  1586.77    1585.77    1585.65     0.12     581 -    593    0   YFESSGMTEHQDR
  1858.02    1857.01    1856.87     0.15     671 -    686    0   HQSTLQEDQFPSADVR
  2215.23    2214.22    2214.15     0.07     979 -    998    0   ILTAFSSLQHLDLDALSENK
  2713.46    2712.46    2712.36     0.10     344 -    369    0   SLQDTYGAEPAGPDGILVEVDLVQAR
  2845.39    2844.38    2844.35     0.04      93 -    116    0   EAYANIAELDQYVFQDSQLEGLSK
No match to: 913.58, 1039.62, 1109.68, 1243.75, 1386.79, 1403.85, 1446.86, 1515.88, 1545.85, 1550.90, 179
```

Fig. 12. Mascot database search results from a tryptic digest of ubiquitinated CIITA, showing a “hit” for a potential ubiquitinated peptide. This potential ubiquitination site has not yet been confirmed by MS/MS data.

have come from another peptide in the mixture, this ubiquitination site cannot be confirmed without MS/MS sequence data of this peptide.

If MS/MS data has been acquired and searched, as is the case when LC/MS/MS has been used, the identity of the peptide can be confirmed and site of ubiquitination found from the database search results (*see* **Note 19**). This was the method used to find the ubiquitination sites in the Peng, *et al.* paper ***(17)***. Unfortunately, this approach often fails where lower amounts of biological material are available. Very low levels of the modified peptide mean that there may not be sufficient intensity of the modified peptide to trigger this automatic data-dependent MS/MS sequencing.

In this case, another much more time-consuming option is to examine the MS spectra to search for a peptide shifted by the masses corresponding these possible modifications. This can be done manually by creating a list of expected "normal" peptide masses, calculating the modified masses, and examining the MS spectra obtained during the LC/MS/MS run. Obviously, at 1 s per scan, thousands of spectra are obtained throughout the course of an extended LC/MS/MS run. Current software systems allow the combination of groups of spectra, and these groups of combined spectra can be examined. Most current software packages also allow the deconvolution of the spectra to singly-charged species, which reduces the complexity of the manual data analysis, since multiple charge states of the possible peptides are deconvoluted to singly-charged species. A semi-automated approach to this task is to combine all of the spectra, perform a deconvolution on the MS data to generate a pseudo-singly charged spectrum (*see* **Note 20**), and then submitting this data to the database search software for searching as an MS data file. As above, the MS data can then be searched for modified peptides (*see* **Note 21**).

If a possible modified peptide is identified in this manner, it is useful to examine the original MS data to see if the calculated +2 and +3 charge states for this peptide co-chromatograph. Now that the masses are known, it is possible to perform a different type of MS scanning, where the precursor ion is specified – in Micromass software, it is called "include only" MS/MS. In this scan mode, MS/MS is only performed on the preselected precursor ions. In a previous study ***(31)***, we have found that this can lead to an increase in sensitivity of a factor of 50–100 for these ions.

Although this scanning mode is dependent on intensity-based triggering, and thus has the same sensitivity limitations as the "survey scan" mode, knowing these characteristic fragment ions also allows the possibility of "precursor ion scanning". Here, when a preset characteristic ion is detected, the data system switches to the MS mode, detects the precursor, and collects the MS/MS data. Similar approaches are already commonly used, for example, in order to find peptides containing acetylated lysine from the acetylated lysine immonium ion ***(32)*** or to find phosphotyrosine-containing peptides from its characteristic immonium ion ***(33,34)***.

The identification of the specific and characteristic fragment ions which we have described above, provides a powerful new approach for finding ubiquitinated peptides. Searching the MS/MS chromatograms (*see* **Note 21**) for these characteristic ions (which should cochromatograph because they are fragment ions from the same peptide) should allow one to find peptides containing ubiquitin side chains.

Since we first suggested the use of extended ubiquitin tags to improve the detection of ubiquitinated peptides in 2005 ***(35)***, two other groups have used the LRGG tag and have demonstrated the improvement resulting from inclusion of this second mass shift. Jeon, et al. ***(36)*** used GG and LRGG mass shifts resulting from GG and LRGG tags to search LC/MS/MS data in a mouse heart digest. The use of the GG tag resulted in the identification of 27 ubiquitination sites on 21 proteins. The use of the LRGG tag resulted in the identification of an additional 6 sites. These two tags were also used to identify the polyubiquitination sites on ubiquitin itself (in this case, Lys 48 and Lys 63). One important observation by this group is that the ubiquitinated proteins from mouse heart were insoluble in detergent-free buffer – CNBr cleavage resulted in smaller more soluble proteins, while preserving the ubiquitination.

Recently the Figey group used both GG and LRGG tags to increase the detection of ubiquitinated peptides from a human MCF7 breast cancer cell culture ***(37)***. This group found a total of 96 ubiquitination sites. Of these sites, 53% were found using internal GGK residue, an additional 8% from the internal LRGGK residue, and 39% from C-terminal GGK peptides (which were cleaved by trypsin at the modified residue). These C-terminal peptides (with the standard tryptic cleavage after K) would, of course, have been detected by the database searches, since at least the 1 missed cleavage expected from blocked tryptic would have been allowed. However, the authors noted that no C-terminal LRGG peptides were detected, only C-terminal GGK peptides. This provides support for the hypothesis that the missed cleavage of the LR-GGK bond results from steric hindrance of the trypsin.

A “two-stage” affinity purification strategy was used by the Figeys group for the study of the ubiquitination of proteins co-purifying with valosin-containing protein (VCP), a ubiquitin-dependent chaperone ***(38)***. In this study, first immobilized anti-VSP and then anti-ubiquitin beads were used. Using this strategy, along with a high-pressure device to improve tryptic digestion efficiencies, 27 ubiquitination sites were mapped on 21 proteins, and 58 additional “probably-ubiquitinated” proteins were also identified by LC/MS/MS, using the 114 or 383-Da mass shift associated with the GG- or LRGG-modified lysine residues.

3.2.6. Determination of Ubiquitin Branched Structure

Polyubiquitinated chains can be thought of as multiple ubiquitinations of ubiquitin. Tryptic cleavage of these linked ubiquitins results in GGK and LRG-GK-tagged peptides from ubiquitin. A detailed strategy for determining this branching structure has been reported by Kaiser and Wohlschlegel ***(39)***, who used the GGK tagged peptides. They reported that they preferred to perform LC/MS/MS or LC/LC/MS/MS on digests of gel-separated proteins because if a mixture of proteins was present, it could not be determined which *protein* these polyubiquitin fragments came from. Also, without separation of the ubiquitinated peptides from the protein (prior to digestion of the polyubiquitin tail), the "average" branch structure would be determined if there were more than one ubiquitin site on each protein. This, or course, would be difficult to accomplish because enzymes that would separate the ubiquitination sites on the protein would also cleave the polyubiquitin chains.

3.2.7. Quantitation of Ubiquitination

Several recent papers have reported different methods of quantifying the ubiquitination at various sites in the protein, as well as quantitation of the various polyubiquitin isoforms. Cripps et al ***(40)*** used targeted detection of ubiquitinated peptides from a targeted protein, and also performed quantitation determination of the ubiquitinated peptides. *Relative* quantitation using selected reaction monitoring (*see* **Note 22**) was performed on GGK-modified tryptic peptides from Tau protein, immunoprecipitated from Alzheimers brain tissue, using unmodified Tau peptides as internal standards. From this MRM data, the authors determined the relative ubiquitination levels of the 3 sites found in this protein. Although the possible formation of the LRGG tag waas not considered in this studym, or any of the quantitation studies described below, it probably would not significantly affect the results.

Gygi and coworkers have recently published two ubiquitination studies with *absolute* quantitation (AQUA ***(42)***; *see* **Note 23**), the first on the epidermal growth factor receptor (EGFR) ***(41)***, and the second on cyclin B1 ***(43)***. In the EGFR study, recombinant EGFR receptor was purified, and LC/MS/MS was performed. Six ubiquitination sites (GGK) were identified, and quantitative determination of the "average" branch structure of the polyubiquitin chain at each site was performed by comparison of the selected reaction monitoring peak areas of each peptide with those from stable-isotope labeled peptides (the "ub-AQUA" method ***(42)***).

This "ub-AQUA" method was also used by the Gygi group to determine the branch structure of ubiquitinated cyclin B1 ***(43)***. The absolute amounts of each of the 10 possible polyubiquitin cleavage products (7 branched and 3 unbranched) were determined by comparison with the amounts of 10 synthetic isotopically-labeled analogues. (For example, the heavy leucine in one of the reference peptide contained six ^{13}C's and one ^{15}N). As in the study by Kaiser and Wohlshlegel described above ***(39)***, ubiquitinated cyclin B1 was first separated by gel electrophoresis, and the quantitation was performed on the extract containing the in-gel digested peptides. Calculations were performed to determine the amount of ubiquitin in long chains vs. short chains.

To determine the relative quantitation of the branch structures in a set of 5 ubiquitin-conjugating enzymes and ubiquitin ligases, the Gygi group used a different strategy, utilizing a set of mutant ubiquitins, each having all but one of the 7 lysines replaced by alanines ***(44)***. The relative amounts of each type of linkage was based on spectral counting ***(45)*** of the resulting GGK-containing peptides, *i.e*. the number of spectra observed in a data-dependent LC/MS/MS acquisition, rather than the abundance of the spectra.

3.2.8. Emerging Strategies

In addition to new quantitative studies, there have been several interesting studies designed to improve detection of ubiquitinated peptides which is still extremely challenging when the amount of biological material is limited. As mentioned above (*see* **Subheading 3.1.2.3.1**), ubiquitinated peptides differ from "standard" peptides in that they have 2 N-termini.

Recently, Cotter and his group have developed a new method which uses this feature ***(46,47)***. This method is based on a derivatization procedure, similar to the chemistry behind Edman sequencing ***(48)***. This reagent attaches an SPITC (4-sulfophenyl isothiocyanate) moiety to the N-termini – to *both* N-termini for ubiquitinated peptides. Under MS/MS conditions, ubiquitinated peptides can then be distinguished from nonubiquitinated peptides by the loss of *two* SPITC groups forming a set of "signature" ions resulting from complete or partial loss of one or two of these tags ("normal" peptides can only have *one* loss of SPITC) (**Fig. 13**). This derivatization procedure, first developed by Keough et al ***(49)***, produces a y series from a derivatized peptide, while reducing the b series (ions which contain the N-terminus). The Cotter group first modified this reagent for more efficient use in aqueous media ***(50)***, and then used it to derivatize tryptic peptides prior to MALDI-MS and MALDI-MS/MS. They used this method for determination of ubiquitination sites in a synthetic peptide containing a GGK tag, and to tetraubiquitin ***(46)***. In a subsequent paper ***(47)***, the Cotter group used this procedure to find the ubiquitination sites on His-tagged CHIP (*C*-terminal *H*SP70-interacting protein), which was affinity purified using Cobalt beads, and IMAC. Three peptides were identified as doubly-tagged based on their MS/MS spectra, which produced "signature" ions from the SPITC moieties and

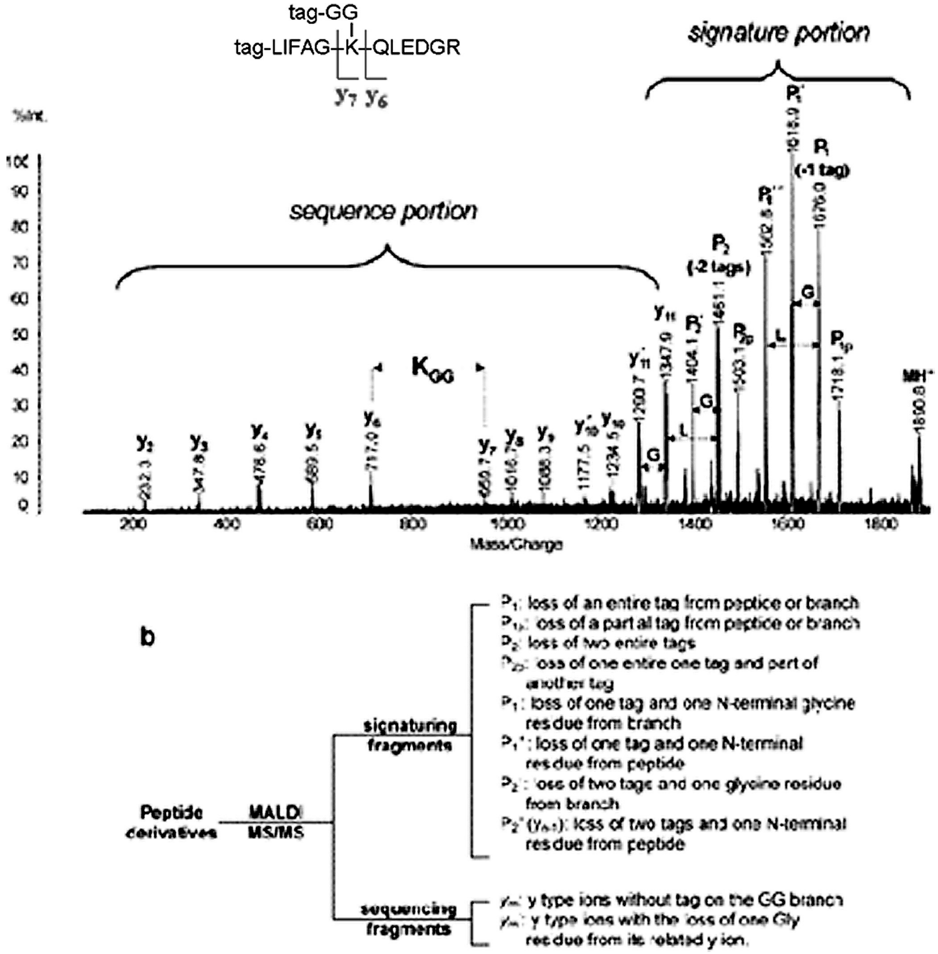


Fig. 13. Fragmentation of an SPITC-labeled ubiquitinated peptide. (Reprinted from ***ref. 46,*** with permission)

sequence ions which were used to identify the site of ubiquitination. In a recent report, the Perales group ***(51)*** used the SPITC approach, and reported improved quality of the resulting MS/MS sequencing, which is another advantage of this approach.

A different method for N-terminal derivatization was recently suggested by the Gebler group ***(52)***. Like the SPITC method, this is a new look at an older derivatization technique for mass spectrometry ***(53)***. This method relies on derivatization of the N-terminal peptides with tris(2,4,6-trimethoxyphenyl) phosphonium acetic acid N-hydroxysuccinimide ester (TMPP-Ac-OSu), which introduces a fixed charge on the N-terminus and simplifies MALDI-.MS/MS sequencing. This method also has potential for improving the detection of "double N-terminal" ubiquitinated peptides.

These N-terminal labeling approaches should be very useful for identifying ubiquitinated peptides from a mixture, especially if the label could be combined with an affinity capture moiety such as biotin. This approach is proving extremely useful for the identification of crosslinked peptides ***(54)***, and it would seem that a similar strategy could be developed for the labeling and recovery of ubiquitinated peptides.

4. Notes

1. We call this "direct" analysis because the affinity beads are placed directly on the target, in contrast to methods were the affinity-bound proteins are eluted first, and the eluate is spotted on the target.
2. If binding has to be done through protein A, or the antibody is dissolved in ascites, the antibody can be crosslinked to the beads ***(55)*** before the target protein is affinity-bound.
3. Since only a small number of beads will be placed on the MALDI target, it is important to get much protein as possible on each bead, so use a small amount (~20 µL) of settled affinity bead slurry.
4. To avoid releasing parts of the antibody into the solution, try to avoid reducing agents such as β-mercaptoethanol or dithiothreitol (DTT) in the binding buffer.
5. Most salts will be removed during the wash steps. Other components not compatible with mass spectrometric analysis (such as glycerol or detergents) should be avoided or minimized. Although Zwittergent is thought to be compatible with mass spectrometry, it seems to make agarose beads turn "gummy," so it should not be used.
6. To recrystallize α-cyano 2-hydroxycinnamic acid, make a saturated solution of α-cyano 2-hydroxycinnamic acid in boiling methanol. Pour off the solution and discard it. Add more methanol, and again make a saturated solution in boiling methanol. This time, pour off the methanol and save it. Evaporate the methanol to dryness in a hood, while protecting the solution and the crystals from light with aluminum foil. Store in the dark or in a vial wrapped with aluminum foil.
7. The matrix solvent must contain both organic and acid so that it dissociates the affinity bound protein from the antibody on the MALDI target.
8. Also known as *Staphloccus aureus* V-8 protease.
9. The antibody used should be covalently attached to the beads, and DTT should not be used (or used in a very low concentration ***(56)***) in the purification step. If the disulfide bridges in the antibody are reduced, the

antibody can be enzymatically digested along with the attached protein. This will lead to a high background of IgG peptides along with the peptides from the target protein, and will make finding the modified peptide more difficult.

10. For proteins affinity-bound to antibody beads, much higher ratios of enzyme (e.g., 5:1) to substrate should be used than for proteins in solution ***(30)***.
11. Be sure to add enough digestion buffer so that the beads can "slosh around" in the Eppendorf tube and don't dry out during the overnight digestion.
12. If you do not know the protein concentration, use ~2 μL of a 1 μg/μL solution.
13. If the sample to be digested is already dissolved in water or a buffer such as PBS, add enough ammonium bicarbonate to the solution to ensure that the pH will be ~7–8.
14. The specificity of gluC depends on buffer used and pH of the solution. Cleavage can be either C-terminal to glutamic acid (in ammonium acetate at pH 4.0) or (ammonium bicarbonate at pH 7.8), or to *both* glutamic and aspartic acid (in PBS, pH 7.8). For a solution digest, you can add a second buffer to the original buffer in order to adjust the pH, but be sure to take both enzymes into account when calculating the expected of the peptide molecular weights.
15. This requires a site license for Mascot, although the company says that if there is sufficient interest, they will add new modifications to their on-line website (www.matrixscience.com).
16. Programming in these modification means, in effect, telling the software that there are three additional types of lysines, with new masses: 242.1374 for GGK, 511.6294 for LRGGK, and 1430.8824 for STLHLVLRLRGGK.
17. In searching using Mascot, first search with no variable modifications, *then* select the target protein and search in the error tolerant mode, specifying the appropriate ubiquitination modifications you have previously entered.
18. Be sure to specify at least two missed cleavages. Cleavage is not expected to occur at the modified lysine.
19. The database search can also be "forced" to consider a specific target program. In MASCOT, this is done by adding the accession number from the appropriate database as the first line of the peak list being searched. (e.g., accession=XXXXX, followed by a blank line).
20. For Micromass MassLynx software, this is MaxEnt 3, under "Tools".
21. If multiple levels of MS/MS spectra are produced, as in the Micromass MassLynx software, *all* of the MS/MS functions must be searched.
22. Multiple reaction monitoring (or selected reaction monitoring) is a technique performed on a tandem mass spectrometer. In this technique, a both the precursor and the product ion (formed in a collision cell) are selected, which makes it a very selective technique for quantitation.

23. This is a confusing term, partly because the AQUA acronym is used by two groups (both from Harvard) for different things, *a*utomated *qua*ntitation ***(57)*** and absolute quantitation ***(42)***. AQUA as used now in mass spectrometry ***(42)*** is a new name for a mass spectrometric technique dating back at least to the early 1970s ***(58, 59)*** where a stable-labeled form of the target analyte is introduced into the sample to act as an internal standard. This isotopically labeled molecule has properties nearly identical to the target analyte, but has a different mass (because of the isotopic label). This allows it to be distinguished from the native form by mass spectrometric analysis.

Acknowledgments

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Detection of Sumoylated Proteins

Ok-Kyong Park-Sarge and Kevin D. Sarge

1. Introduction

Small ubiquitin–related modifier (SUMO) was discovered as a modifier of mammalian proteins in 1997 ***(1–2)***. SUMO has since been demonstrated to be a modifier of many important proteins, giving this modification a vital role in modulating a large number of important cellular processes ***(3–5)***. SUMO proteins are very similar to ubiquitin structurally, but sumoylation does not promote degradation of proteins and instead regulates key functional properties of target proteins. These properties include subcellular localization, protein partnering, and transactivation functions of transcription factors, among others ***(3–5)***. Protein sumoylation plays a particularly vital role in regulating many important processes occurring in the nucleus, and although sumoylation can be found on proteins that exist in a number of cellular compartments, most of the sumoylation characterized to date occurs on nuclear proteins ***(3–5)***. Indeed, proteins of the SUMO conjugation machinery have been found to be localized to nuclear pore complexes, in addition to other locations in the nucleus.

SUMO proteins are covalently attached to lysine residues of proteins, which are generally found within the consensus motif ΨKXE where Ψ is a hydrophobic amino acid and X is any residue. Like ubiquitination, the covalent attachment of SUMO to other proteins involves a series of enzymatic steps (*see* **Fig. 1**), but the proteins involved are distinct from those in the ubiquitin conjugation pathway. First, the SUMO proteins have to undergo proteolytic processing near their C-terminal end to form the mature proteins, a step which is performed by SUMO proteases (Ulps). These proteases are dual-functional, as they are also responsible for cleaving SUMO groups from substrate proteins by cleaving the isopeptide bonds by which they are joined ***(3–5)***. The mature processed SUMO protein is covalently attached via a thioester bond to the Uba2 subunit of the heterodimeric SUMO E1 activating enzyme in an ATP-dependent reaction ***(6–9)***.

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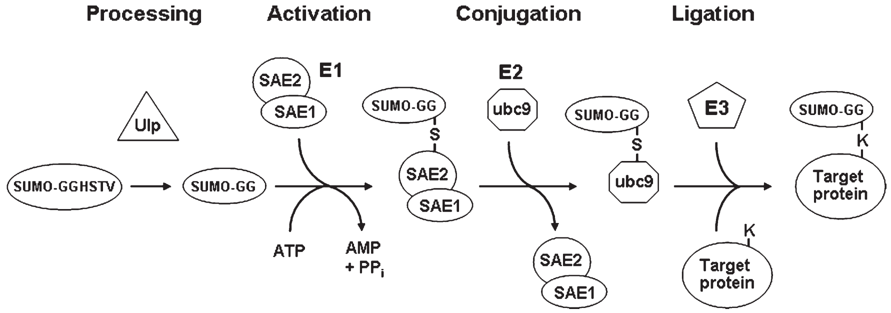


Fig. 1. The SUMO conjugation pathway. After they are translated, SUMO proteins must first be processed by a SUMO protease such as Ulp1, which removes four C-terminal residues so that the mature form ends with a glycine. These SUMO proteases are also responsible for removing SUMO groups from proteins. This mature form is then activated in an ATP-dependent manner by forming a thioester bond with a cysteine residue in the SAE2 subunit of the heterodimeric E1 activating enzyme. Following this activation step, the SUMO moiety is transferred to the E2 conjugating enzyme ubc9. In the final step SUMO is transferred in a ligation reaction from ubc9 to substrate proteins, forming an isopeptide bond between the terminal glycine on SUMO and the ε-amino group of a lysine in the target protein. The efficiency of sumoylation of some proteins is enhanced by SUMO ligase E3 proteins, via their ability to bind both ubc9 and the target protein, thereby increasing the kinetics of the SUMO transfer.

The SUMO moiety is transferred from the E1 to ubc9, the SUMO E2 enzyme, which then binds to the ΨKXE consensus sequence in target proteins and forms an isopeptide bond between the ε-amino group of the lysine within this sequence and the carboxyl group of the C-terminal glycine of the SUMO polypeptide ***(10–13)***. SUMO E3 proteins have been identified that enhance the efficiency of SUMO attachment by interacting with both ubc9 (the E2 enzyme) and the substrate, thereby acting as bridging factors ***(3–5)***. Vertebrate cells contain three SUMO paralogs. SUMO-2 and SUMO-3 are very similar to each other in sequence, and have approximately 50% sequence identity with SUMO-1, which is the best characterized of the three vertebrate SUMO proteins.

In this chapter we describe two different experimental approaches for determining whether a specific protein is sumoylated. One method employs immunoprecipitation of the protein of interest, either endogenous or transfected epitope-tagged protein, followed by Western blot with SUMO antibodies. The sond method involves incubating the protein, either as a ^{35}S-labeled in vitro translation product or purified recombinant protein, with a reconstituted in vitro sumoylation enzymatic reaction, followed by SDS-PAGE and autoradiography or Western blot, respectively, to look for the appearance of higher molecular weight bands indicative of sumoylation. By comparing wild type protein constructs with those containing non-sumoylatable arginine substitutions of candidate target lysine residues, these protocols can also allow identification of those lysine residues where SUMO attachment actually occurs in a given protein. This information then provides the critical reagents for testing the functional consequences of blocking sumoylation of that particular protein. To illustrate the types of data that can be obtained using these methodologies we present figures showing the results of immunoprecipitation and in vitro sumoylation analyses of a transcription factor we study called HSF2.

2. Materials

2.1. Detection of Sumoylated Proteins by Immunoprecipitation Analysis

1. Cells expressing the protein of interest.
2. Phosphate Buffered Saline (PBS).
3. Lysis solution: 0.15 *M* Tris-HCl (pH 6.7), 5% SDS, & 30% glycerol.
4. N-ethylmaleimide.
5. Complete protease inhibitor (Roche; Indianapolis, IN).
6. Protein G-sepharose or protein A-sepharose.
7. Primary antibody capable of immunoprecipitating protein of interest (or against epitope tag if analyzing a transfected tagged protein), species-matched nonspecific IgG, and antibodies against SUMO-1, SUMO-2, or SUMO-3 (Invitrogen).
8. SDS loading buffer
9. Polyacrylamide gel electrophoresis equipment and SDS-PAGE solutions.
10. Reagents for immunoblotting and detection (nonfat dried milk for blocking, and ECL or other detection system).

2.2. Detection of Sumoylated Proteins by In Vitro Sumoylation Analysis

1. Plasmid containing open reading frame of protein of interest oriented to be expressed from a T7 promoter in the vector.
2. pGEX-SUMO-1, pQE30-SUMO-1, pGEX-Ubc9, and pGEX-SAE2/SAE1 bicistronic expression construct, or purified SUMO, ubc9, SAE1/SAE2 available commercially (e.g., LAE Biotech International).
3. Ampicillin and LB media.
4. Isopropyl-β-D-thio-galactopyranoside (IPTG).
5. Phenylmethylsulfonyl fluoride (PMSF).
6. Glutathione-agarose and Ni-NTA-agarose.
7. In vitro translation kit (e.g., Promega TNT T7 Quick for PCR DNA kit).
8. Polyacrylamide gel electrophoresis equipment and SDS-PAGE solutions.
9. Whatman paper, X-ray film, and cassettes for detection of ^{35}S-labeled proteins in sumoylation reaction.

3. Methods

3.1. Detection of Sumoylated Proteins by Immunoprecipitation Analysis

In this protocol, proteins to be tested for sumoylation are immunoprecipitated using lysis buffers designed to block the action of desumoylating enzymes (*see* **Note 1**) and then subjected to Western blot analysis using anti-SUMO antibodies to look for the appearance of a band with a size consistent with a sumoylated form of the protein. Although the theoretical molecular weight of the SUMO proteins is approximately 11 kDa, the size increase for each SUMO added on SDS-PAGE is typically in the range of 15–17 kDa. In the case of a protein with multiple sumoylation sites, or where SUMO chains form on a lysine target site (*see* **Note 2**), multiples of this size increase are expected, sometimes yielding very large shifts in mobility. Multiple bands representing different sumoylation states of the protein are also possible. This approach can be used to analyze endogenous proteins, or transfected proteins containing an epitope tag (FLAG, myc, etc.) (*see* **Note 3**). The transfection approach can also be used to determine the lysine residue(s) to which the SUMO group is attached, by comparing the sumoylation of wildtype constructs to ones in which candidate lysines have been changed to non-sumoylatable arginines. Two different lysis conditions are described, one using SDS to inhibit desumoylase enzymes and the other containing N-ethylmaleimide, a chemical inhibitor of these enzymes.

1. Tissue culture cells are grown in media appropriate for that cell line or primary cell type, and typically at least 1×10^6 cells are needed for each immunoprecipitation.
2. For harvesting place the plate of cells on ice, remove media by aspiration and add 1 ml of ice-cold PBS to the plate. Scrape cells off plates with a cell scraper, transfer to a 1.5 ml microcentrifuge tube.
3. Collect cells by centrifugation at 13,000 ×g for 30 sonds at room temperature, and remove supernatant by aspiration.
4. Lyse cells in 150 µl of lysis solution which is then diluted 1:10 in PBS/0.5% NP40 plus complete protease inhibitor (Roche) and centrifuged (16,000 g, 10 min, 4°C) to remove cellular debris. Sonication can be done prior to the centrifugation step if the lysate is highly viscous. Alternatively, the cells can be lysed in any of the standard immunoprecipitation lysis buffers (e.g., RIPA, etc.) known to extract the protein of interest, providing the lysis solution is supplemented with 20 m*M* N-ethylmaleimide (desumoylase inhbitor, freshly dissolved).
5. While the cell lysate is being centrifuged prepare 30 µl of 50% protein G-Sepharose (or protein A-sepharose if that is preferential for the particular antibody) as per manufacturer's instructions. After protein G-Sepharose has been prepared add the above cell lysate to the protein G-Sepharose and rotate at 4°C for a half hour to preclear the lysate.
6. Pellet the protein G-Sepharose by centrifugation (16,000 g, 20 s, 4°C) and transfer the supernatant to a new tube. At this point take 30 µl of cell lysate, place in a separate tube with 10 µl 4×SDS load buffer and label "input."

7. Divide the remainder of each treatment lysate into 2 equal amounts in separate 1.5 ml centrifuge tubes. To one of the tubes add a sufficient amount of primary antibody and to the other tube add a species-matched nonspecific IgG. Place samples on rotator at 4°C for 1 hr after which add 20 µl of PBS washed protein G-Sepharose and rotate at 4°C for 3 h.
8. Collect beads by centrifugation (16,000 g, 10 sonds, 4°C) and discard supernatant.
9. Wash the beads 4 times with PBS/0.5% NP40 plus complete protease inhibitor, or other lysis buffer if another was chosen, collecting the beads by centrifugation after each wash (16,000 g, 10 s, 4°C). Add 30 µl 2 × SDS-PAGE loading buffer to beads after removing supernatant from final wash.
10. Separate immunoprecipitated proteins by SDS-polyacrylamide gel electrophoresis.
11. Transfer proteins to nitrocellulose or nylon membrane and subject to Western blotting using antibodies against SUMO-1, SUMO-2, or SUMO-3 (commercially available). This methodology can also be used to examine the sumoylation state of an epitope-tagged version of the protein of interest being expressed in transfected cells (*see* **Note 3**). The results of immunoprecipitation analysis to examine sumoylation of the HSF2 protein is shown in **Fig. 2**.

3.2. In vitro Sumoylation Assay

In this protocol the protein of interest is in vitro translated (typically with ^{35}S-methionine incorporation) and then incubated in a reaction containing the SUMO E1 and E2 enzymes and SUMO-1, followed by SDS-PAGE and autoradiography to determine whether a lower mobility band appears that would be consistent with a sumoylated form of the target protein. Because of the high concentrations of SUMO E1 and E2 enzymes used in this in vitro sumoylation reaction, the need for SUMO E3 proteins is diminished and thus sumoylation can be detected without their addition. Performing the in vitro sumoylation reaction using mutants of the protein in which candidate sumoylation site lysine

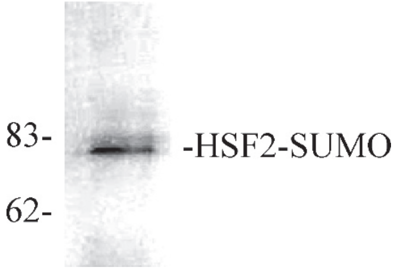


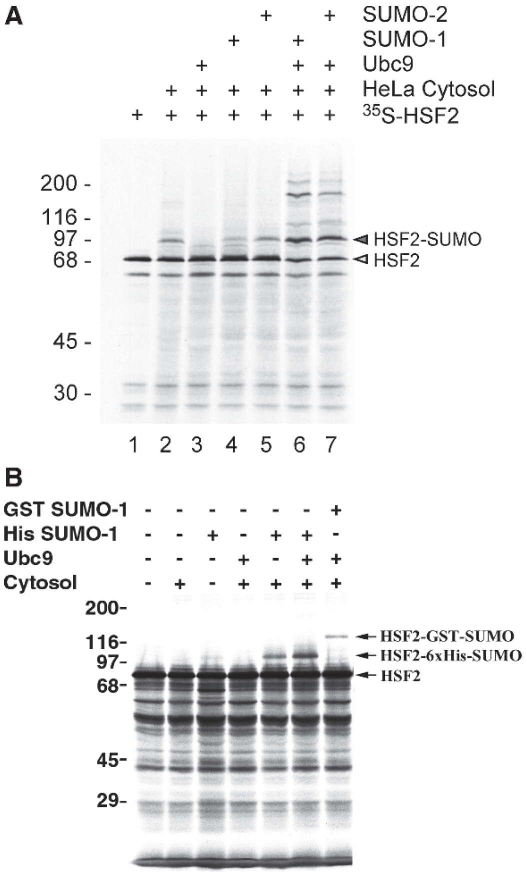
Fig. 2. Detection of sumoylation by immunoprecipitation/SUMO-1 Western blot. HSF2 protein was immunoprecipitated from extracts of HeLa cells followed by Western blot using anti-SUMO-1 antibodies. The positions of molecular weight standards are indicated on the left side of the panel. (Reprinted with permission from ***ref. 15***).

residues are changed to nonsumoylatable arginines can be used to determine the site(s) at which SUMO attachment is occurring (e.g., *see* **Fig. 4**). The expression and purification of the recombinant proteins required for the in vitro sumoylation assay is described in **Subheading 3.2.1**, and the protocol for performing assay itself is described in **Subheading 3.2.3**.

3.2.1. Expression If Recombinant SUMO, ubc9 (SUMO E2), and SAE1/SAE2 (SUMO E1) Heterodimer

The SUMO proteins are expressed and purified as fusion proteins with GST or 6xHis at the N-terminal end of the SUMO proteins, and these affinity tags do not need to be removed prior to using these SUMO proteins for the sumoylation reaction. The size difference of GST-SUMO vs. 6xHis-SUMO can also provide a useful control for the sumoylation reaction as it yields a predictable size difference between the sizes of the sumoylation products (e.g., *see* **Fig. 3**). The SUMO E1 is a heterodimer of SAE1/SAE2, and is active when expressed in E. coli from a bicistronic contruct of GST-SAE2 and untagged SAE1; the two proteins complex and can be purified using glutathione-agarose affinity chromatography ***(14)***. The SUMO E2 can be expressed which appears to be more active, at least in our hands when the GST tag is removed by thrombin cleavage. The following is the general protocol for expressing and purifying these recombinant proteins needed for the *in vitro* sumoylation assay. Once purified these recombinant proteins can be stored at −80°C for extended periods of time.

1. Transform expression construct into DH10B E. coli cells using standard molecular biology methods.
2. Plate cells on LB plates containing ampicillin and incubate overnight at 37°C.
3. Select single colonies and grow overnight at 37°C in LB media containing ampicillin.
4. Inoculate individual liters of LB containing ampicillin with aliquots (5 mL) of overnight culture and grow cells to an O.D.$_{600\,\mathrm{nm}}$0.6–0.8.
5. Induce the cells with IPTG (1 m*M*) for 3 hs.
6. After 3 hr induction harvest cells by centrifugation (4,000 g; 5 min; 4°C) and resuspend pellet in PBS (4°C) then repellet cells. At this point the cell pellet can be extracted or stored at −80°C.
7. To extract protein from the cells they are resuspended in PBS (4°C) with PMSF (phenylmethylsulfonyl fluoride) to a final concentration of 1 m*M*.
8. Pass cells through a French press at 10,000 to 14,000 psi, and repeat to ensure complete lysis.
9. Centrifuge the cell lysate (30,000 g;1 h; 4°C) and retain the supernatant.
10. Purify proteins using standard nondenaturing affinity chromatography techniques suitable for their fusion tags (glutathione agarose affinity chromatography for GST-fusion proteins and Ni-NTA-agarose affinity chromatography for 6 × His-fusion proteins).


A
+ + SUMO-2
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+ + + + + + HeLa Cytosol
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200 -
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HSF2-6xHis-SUMO
HSF2

Fig. 3. Analysis of SUMO-1 modification by reconstituted in vitro sumoylation reaction. **(A)** In vitro translated ^{35}S-labeled HSF2 protein was incubated with HeLa cytosol (as a source of E1), Ubc9, SUMO-1, SUMO-2 or with various combinations of each of these, and then subjected to SDS-PAGE followed by autoradiography. The positions of unmodified and SUMO-modified HSF2 are indicated to the right of the panel. **(B)** In vitro translated ^{35}S-labeled HSF2 protein was subjected to the in vitro SUMO-1 modification assay using either 6xHis-SUMO-1 or GST-SUMO-1 as the SUMO-1 substrate for the reaction. (Reprinted with permission from ***ref. 15***).

11. Check purified proteins by Coomassie blue staining of a SDS-PAGE gel (GST-SUMO1 = 38 kDa, GST-Ubc9 = 44 kDa, and 6His-SUMO1 = 14 kDa).
12. For the *in vitro* sumoylation assay the GST-Ubc9 needs to be thrombin cleaved to remove the GST-tag. This can be done following standard protocols. Check thrombin cleavage of GST-Ubc9 by Coomassie staining of a SDS-PAGE gel (Ubc9 = 18 kDa).

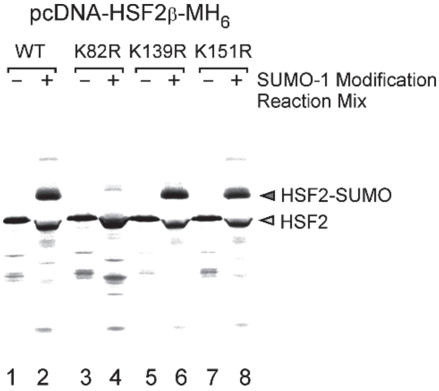

Fig. 4. In vitro translated ^{35}S-labeled wildtype HSF2 protein and the HSF2 SUMO-1 consensus site mutants K82R, K139R, and K151R were used as substrates in *in vitro* SUMO-1 modification reactions. The positions of unmodified and SUMO-modified HSF2 proteins are indicated to the right of the panel. (Reprinted with permission from ***ref. 15***).

3.2.2. In Vitro Sumoylation of Rabbit Reticulocyte System Translated Proteins

The in vitro sumoylation assay uses ^{35}S-methionine radiolabelled protein as the substrate. We generate radiolabelled protein using the TNT T7 Quick for PCR DNA kit (Promega, Madison WI) following the manufacturers instructions. Described below is the SUMO modification procedure to be utilized with radiolabelled translated proteins.

1. Translate target protein fresh before each sumoylation assay following the manufacturer's instructions. Place freshly translated protein on ice until **step 3**.
2. For each sumoylation reaction prepare a mixture containing the following (on ice): 1 µl 10× sumoylation buffer, 0.4 units creatine phosphokinase, 10 m*M* creatine phosphate, 0.6 units inorganic pyrophosphatase, 100 ng purified SAE1/SAE2 heterodimer (E1), 400 ng purified ubc9 (E2), 1 µg purified 6 × His-SUMO-1, and H_2O to 10 µl. 10× sumoylation buffer contains 500 m*M* Tris-HCl (pH 7.6), 500 m*M* KCl, 50 m*M* $MgCl_2$, 10 m*M* DTT, and 10 m*M* ATP. As a negative control, make another mix identical to the one above except that it lacks SUMO protein. If desired a third mix can be made in which GST-SUMO-1 is added instead of 6 × His-SUMO.
3. Set up the reactions (no SUMO control, +6 × His-SUMO, and +GST-SUMO) by adding 2 µl in vitro translated protein to the 10 µl reaction mixes, and then incubate at 37°C for 1 hr.
4. Terminate the reaction by adding 12 µl 2×SDS-PAGE load buffer. Store at −20°C until gel electrophoresis.
5. Boil the samples for 5 min and separate on SDS-PAGE gel. After electrophoresis the gel is fixed for 10 min in SDS-PAGE fixing solution (50% methanol/10% acetic

acid), then dried on Whatman paper, and finally placed on x-ray film. ^{35}S emissions are low energy and so it is advisable not to leave plastic wrap between the dried gel and the X-ray film if possible, as this can increase required exposure times.

6. One important experiment to do to give confidence in the in vitro sumoylation assay is to do a reconstitution test where each of the components required for sumoylation (E1, ubc9, SUMO-1) are individually left out of the reaction and compared to a reaction where all components are present **(Fig. 3A)**. As shown in this figure, such an experiment can also reveal the relative efficiency of sumoylation of your target protein by different SUMO proteins (e.g., SUMO-1 vs. SUMO-2). Another experiment which increases confidence that your protein is indeed being sumoylated vs. being targeted by some other modification is to compare the effect of using of 6×His-SUMO vs. GST-SUMO as the donor SUMO, because this gives a predictable size shift between the sumoylated reactions **(Fig. 3B)**.
7. To identify the sumoylation site in the protein of interest site directed mutagenesis is done to change candidate lysine (changed to arginine) in the sumoylation consensus sequence (ΨKXE) to non-sumoylatable arginine residues utilizing protocols such as the Quickchange Site Directed Mutagenesis Kit (Stratagene). The extent of sumoylation of the wild type vs. lysine-to-arginine mutant can then be compared using either the transfection/immunoprecipitation approach or the in vitro sumoylation assay. Results of such an experiment using the in vitro sumoylation assay are shown in **Fig. 4**.

4. Notes

1. SUMO-modified proteins are highly susceptible to SUMO proteases. The SDS in the lysis buffer described in this protocol inactivates the SUMO proteases allowing for easier detection of sumoylated proteins. However, a common complication with the SDS-lysis method is that the cell lysates tend to be very viscous and sticky due to genomic DNA in the lysate. This problem is remedied by brief sonication which shears the DNA and makes the samples easier to manipulate. SUMO proteases can also be inhibited by the addition isopeptidase inhibitor N-ethylmaleimide (20 mM) to standard lysis buffers such as NP-40 lysis buffer (50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 1% NP-40) if another lysis buffer besides the SDS-lysis is more desirable.
2. The size of putative sumoylated forms of a protein on SDS-PAGE will depend on how many different SUMO attachment sites the protein has. In addition, SUMO-2 and SUMO-3 have been reported to form polymeric chains reminiscent of ubiquitin, which could result in large increases in size for a sumoylated protein on SDS-PAGE compared to the non-sumoylated form ***(14)***. Thus, it is possible to observe bands that are multiples of the approximate 15–17 kDa size of each SUMO unit, as well as multiple bands representing different sumoylation states.

3. Investigating sumoylation may also be done using cells transfected with fusion-tagged plasmid constructs of the protein thought to be sumoylated with immunoprecipitation utilizing fusion tag antibodies which are available from commercial sources (e.g., GFP, FLAG, Myc, and 6×His tags). In these types of experiments it is advisable to co-transfect the cells with a SUMO expression construct (often this is epitope-tagged) to ensure that sufficient SUMO protein is present in the transfected cells to allow for efficient sumoylation of the transfected target protein being tested.

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Efficient Enrichment of Intact Phosphorylated Proteins by Modified Immobilized Metal-Affinity Chromatography

Anna Dubrovska

1. Introduction

The lack of an efficient technique to enrich for phosphorylated proteins is limiting of phosphoproteomes studies. Enrichment with antiphospho serine, antiphospho threonine, or anti-phospho tyrosine antibodies depends on the affinity and specificity of antibodies that limit comprehensiveness of the analysis ***(1–3)***. Metabolic labeling of cells with inorganic (^{32}P) phosphate, followed by an analysis of the whole proteome, requires efficient separation to exclude comigration with nonphosphorylated proteins ***(4,5)***. Chemical modifications of phosphorylated residues in the peptides, followed by MS, allow identification of phosphorylation sites ***(1,5–8)***. However, these techniques do not provide information about full length proteins, e.g., molecular mass of intact proteins. Immobilized metal-affinity chromatography (IMAC) has been successfully used for enrichment of phosphopeptides ***(1,5,9,10)***, but the efficiency of purification of phosphoproteins has been low, indicating significant losses of phosphoproteins ***(5,9,10)***. Another important limitation of available IMAC methods is that bound proteins are eluted into a solution which is incompatible with further separation by 2-DE. A modified IMAC technique developed in our laboratory makes possible efficient and comprehensive analysis of phosphoproteins, and is compatible with 2-DE ***(11)***.

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2. Materials

2.1. Cell Culture and Lysis

1. Clonetics® Normal Human Mammary Epithelial Cell Systems (Lonza Walkersville, Inc.).
2. Cell lysis buffer: 1% (v/v) Triton X100, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, protease inhibitors (Roche Diagnostics; complete inhibitor cocktail tablets EDTA-free) (*see* **Note 1**). Store at 4°C. Add protease inhibitors just prior to use.
3. Transforming growth factor-β1 (TGFβ1, Peprotech) is reconstituted with sterile 4 mM HCl containing 1 mg/mL BSA to a final concentration 50 μg/mL and stored in single use aliquots at −80°C, and then added to tissue culture dishes at the concentration 5 ng/mL.
4. Radioactive orthophosphate ((^{33}P) or (^{32}P) orthophosphate) (GE Healthcare Uppsala, Sweden).

2.2. Preparation of Fe-NTA Agarose

1. NTA agarose (Qiagen, VWR International AB).
2. 6 *M* guanidine-HCl in 0.2 *M* acetic acid. Store at room temperature.
3. Water solution of 2% (w/v) sodium dodecyl sulfate (SDS). Store at room temperature.
4. 25%, 50%, 75% and 100% (v/v) ethanol-water solutions.
5. Aqueous solution of 100 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0
6. Solution of 0.1 *M* $FeCl_3$ in 0.1 *M* acetic acid. Prepare just prior to use.
7. Aqueous solution of 0.1 *M* acetic acid.
8. Rotor shaker.
9. Empty econo-pac chromatography columns (Bio-Rad, Hercules, CA).

2.3. Phosphoprotein Enrichment Procedure

1. Washing buffer: 5 m*M* Tris-HCl, pH 6.3, 150 m*M* NaCl. Store at 4°C.
2. Elution buffer: 100 m*M* dithiotriethol (DTT), 2% (w/v) SDS, 50 m*M* Tris-HCl, pH 6.8. Prepare just prior to use.
3. Methanol 100%.
4. Glacial acetic acid.
5. Aqueous solution of 25% (v/v) methanol, 10% (v/v) acetic acid.
6. Quick Start Bradford Protein Assay Kit (Bio-Rad, Hercules, CA).

2.4. 2-DE Electrophoresis

1. Rehydration solution: 8 *M* urea, 2.5% (w/v) CHAPS, 0.28% (w/v) DTT, 0.5% (v/v) IPG buffer, pH 3–10 (GE Healthcare), 0.002% bromophenol blue. Store at −80°C. Add DTT just prior to use.
2. DTT or iodoacetamide-containing equilibration buffer: 2% SDS, 50 m*M* Tris-HCl pH 8.8, 6 *M* urea, 30% (v/v) glycerol, 0.002% bromophenol blue. Store in 10 ml aliquots at −80°C. Add DTT to 1% or iodoacetamide to 2.5% just prior to use.

3. SDS electrophoresis buffer: 25 m*M* Tris, 192 m*M* glycine, 0.1% SDS, pH 8.3. Store at room temperature.
4. Agarose sealing solution: 0.5% agarose, 0.002% bromophenol blue in SDS electrophoresis buffer.
5. 4× resolving gel buffer: 1.5 *M* TrisHCl, pH 8.8. Filter solution through a 0.45 µm filter. Store at 4°C.
6. 10% ammonium persulfate.
7. 10% SDS.
8. Acrylamide/bis acrylamide solution (37.5:1; T = 40%, C = 2.6%).
9. N,N,N,N'- Tetramethyl-ethylenediamine (TEMED) (GE Heralthcare, Uppsala, Sweden).
10. Water-saturated isobutanol. Shake equal volumes of water and isobutanol in a glass bottle and allow to separate. Use a top layer. Store at room temperature.
11. Prestained molecular weight markers (New England BioLabs, Ipswich, MA).
12. IPGDry strips with immobilized pH gradient, pH range 3–10, 18 cm, linear (GE Healthcare, Uppsala, Sweden).
13. Ettan IPGphor first-dimension isoelectric focusing system (GE Healthcare, Uppsala, Sweden).
14. Ettan DALT*six* Electrophoresis System (GE Healthcare, Uppsala, Sweden).
15. ImagePlatinum software (GE Healthcare, Uppsala, Sweden).

2.5. Silver Staining Procedure

1. Fixing solution: 20% (v/v) methanol, 10% (v/v) acetic acid.
2. Sensitizing solution 30% (v/v) ethanol, 0.2% (w/v) sodium thiosulphate ($Na_2S_2O_3$), 6.8% (w/v) sodium acetate (CH_3COONa).
3. Silver solution: 0.25% silver nitrate, $AgNO_3$.
4. Developing solution: 2.5% (w/v) sodium carbonate (Na_2CO_3), 0.015% (v/v) formaldehyde. Add formaldehyde immediately before use.
5. Gel drying solution: 30% (v/v) methanol, 5% (v/v) glycerol.
6. Cellophane sheets.
7. Rocking platform.
8. Fuji ImageReader software (Fuji).
9. FujiX2000 PhosphorImager (Fuji).
10. AIDA 2D Densitometry software (Bio Imaging).

2.6. Western Blotting for Phosphorylated Proteins

1. Criterion precast gel system (Bio-Rad, Hercules, CA).
2. Criterion TrisHCl Gels, 12.5% (Bio-Rad, Hercules, CA).
3. Criterion electrophoresis cell (Bio-Rad, Hercules, CA).
4. Criterion blotter cell (Bio-Rad, Hercules, CA).
5. Hybond P membrane (Amersham Biosciences, Piscataway, NJ).
6. Tris-buffered saline with Tween (TBS-T): make 10× stock solution with 1.37 M NaCl, 27 m*M* KCl, 250 mM Tris-HCl, pH 7.4, 1% Tween-20. Dilute 100 mL with 900 mL water to use.

7. Blocking solution and antibody dilution buffer: 5% (w/v) BSA fraction V in TBS-T.
8. Primary antibody: antiphospho tyrosine; antiphospho threonine (Santa Cruz, CA); anti-phospho serine (Sigma-Aldrich).
9. Secondary antibody: Antimouse IgG conjugated to horse radish peroxidase (GE Healthcare).
10. Chemiluminescence Luminol Reagent (Santa Cruz, CA).
11. Kodak BioMax MR film (Sigma-Aldrich).

3. Methods

The described Fe-IMAC procedure permits significant enrichment of phosphoproteins, and allows generation of good quality 2-D gels. Combined with the simplicity of the procedure described here, it provides a tool for the efficient and comprehensive analysis of phosphoproteins. This technique may be applied to various types of biological material from cultured cells to tissues.

3.1. Preparation of Fe-NTA Agarose

NTA or Ni-NTA agaroses are commercially available from Qiagen and can be used for preparation of Fe-NTA resin using the following procedure:

1. Place the column on a stand above the capacious beaker for flow-through collection. Add 50% NTA agarose slurry in the column on the basis of 1 mL the beads per 1.5 mL of total cell lysate.
2. Wash the column with 2 volumes of 6 *M* guanidine-HCl in 0.2 *M* acetic acid.
3. Wash the column with 5 volumes of ddH2O.
4. Wash the column with 3 volumes of 2% SDS.
5. Wash the column with 1 volume of 25% ethanol.
6. Wash the column with 1 volume of 50% ethanol.
7. Wash the column with 1 volume of 75% ethanol.
8. Wash the column with 5 volumes of 100% ethanol.
9. Wash the column with 1 volume of 75% ethanol.
10. Wash the column with 1 volume of 50% ethanol.
11. Wash the column with 1 volume of 25% ethanol.
12. Wash the column with 1 volume of ddH_2O.
13. Wash the column with 5 volumes of 100 mM EDTA, pH 8.0.
14. Wash the column with 10 volumes of ddH_2O.
15. Wash the column with 5 volumes of 0.1 *M* acetic acid.
16. Wash the column with 5 volumes 0.1 *M* $FeCl_3$ in 0.1 *M* acetic acid.
17. Close the column tip and add 5 volumes 0.1 *M* $FeCl_3$ in 0.1 *M* acetic acid.
18. Close the upper side of column and incubate the NTA beads with 0.1 *M* $FeCl_3$ solution on rotor shaker for 16 h at room temperature.
19. Wash the column with 5 volumes of 0.1 *M* acetic acid. Beads can be stored in 0.1 *M* acetic acid at 4°C for 2 weeks.

20. Wash the column with 5 volumes of lysis buffer (1% Triton X100, 150 m*M* NaCl, 10 m*M* TrisHCl, pH 8.0, protease inhibitors). Washed Fe-NTA beads can be used for phosphoprotein enrichments procedure.

3.2. Metabolic Labeling

To monitor phosphorylated proteins, we performed metabolic labeling of MCF10A human breast epithelial cells with radioactive (^{33}P) orthophosphate or (^{32}P) orthophosphate. (^{33}P) isotope has lower energy, as compared to (^{32}P) isotope, which may be of importance for safety considerations (*see* **Note 2**).

1. The MCF10A cells were growing to confluence in mammary epithelial growth medium (MEGM) in 150 mm dishes.
2. The cells were metabolically labeled with serum free MEGM medium supplemented with 0.25 mCi/mL (^{32}P) inorganic phosphoate for 16 h to ensure an efficient incorporation of radioactive isotope into the proteins.
3. The cell were treated or not with 5 ng/ml TGFβ1 for 3 h to initiate TGFβ1-dependent protein phosphorylation.
4. The cells were washed 3 times with cold washing buffer (*see* **Note 3**).
5. Proteins were extracted from cells using lysis buffer (3 mL of lysis buffer for 15 cm dish). To provide complete protein extraction cell were incubated with lysis buffer on ice for 30 min with agitation and the material was scraped into the appropriate labeled tubes.

3.3. Phosphoprotein Enrichments Procedure for the Cell Culture

It is important to pick up a small amount of each chromatography fraction (30–60 mkl) for the next analysis. Make a note about total volume of each fraction taken for analysis as it might be helpful for estimation of phosphoprotein binding.

1. Cell extracts were clarified by centrifugation at 13000 g for 20 min at 4°C, and were incubated with washed Fe-IMAC beads for 12 h at 4°C on rotor shaker. We applied 3 mL of lysate with average concentration of total proteins 0.85 mg/ml for 2 mL of Fe-IMAC beads directly into the chromatography column (*see* **Note 4**).
2. After incubation with cell extract, Fe-IMAC beads were washed six times with washing buffer. Washing procedure was performed at room temperature. The bound proteins were eluted by incubation with a buffer containing 100 m*M* dithiotriethol (DTT), 2% SDS, 50 m*M* TrisHCl, pH 6.8 for 5 min at 95°C (*see* **Note 5**).
3. Eluted proteins were precipitated by adding of methanol to 25% and acetic acid to 10% directly to the samples followed by incubation on ice for 30 min (*see* **Note 6)**.
4. Precipitated proteins were centrifuged at 13000 g for 30 min at 4°C.
5. The pellet was washed twice with 25% (v/v) methanol, 10% (v/v) acetic acid solution followed by centrifugation at 13000 g for 30 min at 4°C (1 mL of precipitation solution per 1.5 mL tube).

6. The result white protein-containing pellet was dried in vacuum centrifuge to remove traces of a precipitation solution (*see* **Note 7**).
7. The dried protein pellet was dissolved in rehydration solution.
8. Complete solubilization of proteins required 5–12 h vortexing at room temperature; prolonged drying in a vacuum centrifuge increased the time of solubilization.

3.4. Phosphoprotein Enrichments from Tissue

The Fe-IMAC technique described here is also suitable for enrichment of phosphoproteins from tissues.

1. Frozen tissue (0.5- 1 cm^3) was placed in Petry dish in 3 mL of ice-cold lysis buffer and cut into small pieces (1 mm^3) with scalpel.
2. Tissue pieces were transferred in 50 mL Falcon tube and sonicated on ice by performing 10 cycles at 30 s/cycle with 1 min interval between the cycles to cool down the tube content.
3. Tissue extract was clarified by centrifugation at 13000 g for 20 min at 4°C, and protein concentration was measured. 3 mL of the tissue lysate with protein concentration 0.85 mg/mL was incubated with 2 mL of Fe-IMAC beads for 12 h at 4°C on rotor shaker.
4. All the next stages of the procedure are the same as described above for the cell culture. Using this described technique we observed 81% recovery of phosphoproteins extracted from chicken liver, as evaluated by immunoblotting with anti-threonine antibodies (**Fig. 2E**). An example result for 2-DE of phosphoproteins enriched from chicken liver is shown in **Fig. 2F** and **2G**.

3.5. Evaluation the Efficiency of Phosphoprotein Enrichment

To evaluate the efficiency of phosphoprotein enrichment by Fe-IMAC, we performed 1-DE and 2-DE, followed by monitoring of phosphorylated proteins by Western blotting or authoradiography.

3.5.1. Silver Staining Procedure

1. To estimate the efficiency of phosphoprotein binding to and elution from Fe-IMAC beads all fraction of Fe-NTA based chromatography were taken for 1-DE (input, flow-through, washes, elution and combined elution fraction). Combined elution fraction is the resulted protein solution obtained after dissolving the washed protein pellet in rehydration solution.
2. Equal volume of each chromatography fraction (50 mkl) was taken for SDS-PAGE separation using Criterion electrophoresis system.
3. For silver staining the gel was placed in a tray containing fixing solution and agitated on a shaker for at least 2 h.
4. The fixing solution was drained from the tray and the gel was washed ddH_2O for 10 min.
5. The sensitizing solution was added and gel was agitated in sensitizing solution for 30 min.

6. The sensitizing solution was drained from the tray and the gel was washed with ddH$_2$O 4 times for 15 min.
7. The gel was incubated in silver solution for 30 min.
8. After 30 min, the silver nitrate solution was drained into an appropriate waste beaker and gel was washed with ddH$_2$O two times, 1 min each time.
9. The developing solution was added to the gel, and agitated until tiny yellow or brown precipitate appears. Then developer was poured off, add fresh developer was added. The developing step was continued until desired intensity of protein staining is achieved.
10. Adding the fixing solution terminated the staining.
11. The gel was then agitating in drying for 20 min.
12. The soaked gel was placed between the wet cellophane sheets and dried on the gel drier at 65°C for 1 h (*see* **Note 8**).

3.5.2. Estimation a Chromatography Efficiency Using 1-DE Gels

1. To monitor efficiency of phosphoprotein binding FE-NTA chromatography fractions were separated by 1-DE and the gels were exposed in a FujiX2000 PhosphorImager. Examples of the silver stained gel and autoradiography are shown in **Fig. 1A** and **1B**.
2. Quantification of radioactivity associated with proteins separated by SDS-PAGE was performed using Fuji ImageReader software, and was normalized to volumes of fraction using the formula $A_{ppf} = D/V$, where A_{ppf} is an amount of phosphoproteins in each fraction, D is densitometric intensity of this fraction and V is a fraction volume.
3. AIDA 2D Densitometry module (Bio Imaging) was used to evaluate the relative amount of total proteins in each chromatography fraction. Data obtained by densitometry of silver stainined gel and autoradiography were used to calculate an yield of phosphoproteins (Y_{pp}), concentration factor (C_{pp}) and and level of phosphoproteins in the elution fraction (E_{pp}) as shown in **Fig. 1C**.

3.5.3. Monitoring the Phosphoprotein Binding by Western Blotting for Phosphorylated Proteins

1. Criterion electrophoresis system was used for protein SDS-PAGE separation and blotting.
2. Once the transfer was completed, the nitrocellulose membrane was washed with 1× TBS-T with agitation 3 times for 15 min each wash.
3. After washing the membrane was blocked with 5% BSA in TBS-T for 1 h at room temperature on a rocking platform.
4. The blocking buffer was discarded and the membrane was incubated with primary antibody for antiphospho tyrosine (dilution 1:500), anti-phospho threonine (dilution 1:500), antiphospho serine (dilution 1:1000), anti-Smad2 and antiphospho Smad2 proteins. Anti-Smad2 and antiphospho Smad2 antibody were prepared in our institute and used with dilution 1:500. All antibodies were diluted in 5% BSA in TBS-T. The membrane was incubated with primary antibody solution overnight at 4°C on a rocking platform (*see* **Note 9**).

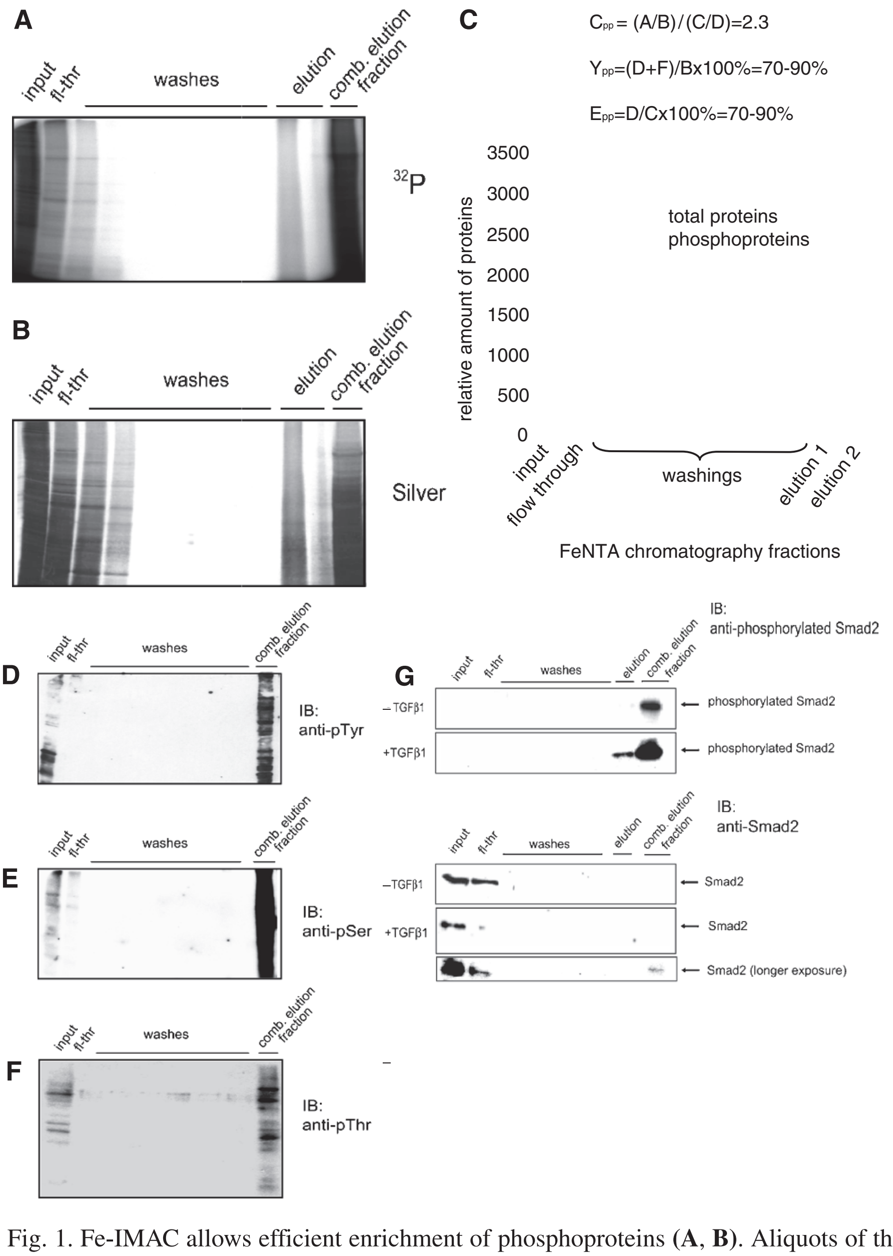
A
input
fl-thr
washes
elution
comb. elution fraction
32P
B
input
fl-thr
washes
elution
comb. elution fraction
Silver
C
Cpp = (A/B)/(C/D)=2.3
Ypp=(D+F)/Bx100%=70-90%
Epp=D/Cx100%=70-90%
relative amount of proteins
3500
3000
2500
2000
1500
1000
500
0
total proteins
phosphoproteins
input
flow through
washings
elution 1
elution 2
FeNTA chromatography fractions
D
input
fl-thr
washes
comb. elution fraction
IB: anti-pTyr
E
input
fl-thr
washes
comb. elution fraction
IB: anti-pSer
F
input
fl-thr
washes
comb. elution fraction
IB: anti-pThr
G
IB: anti-phosphorylated Smad2
input
fl-thr
washes
elution
comb. elution fraction
–TGFβ1
+TGFβ1
phosphorylated Smad2
phosphorylated Smad2
IB: anti-Smad2
input
fl-thr
washes
elution
comb. elution fraction
–TGFβ1
+TGFβ1
Smad2
Smad2
Smad2 (longer exposure)

Fig. 1. Fe-IMAC allows efficient enrichment of phosphoproteins (**A**, **B**). Aliquots of the whole cell lysate (input; 1/50 of the input), flow-through (fl-thr; 1/50 of the fraction), washes, elution fractions from Fe-IMAC beads (elution; 1/50 of the elution fraction), and elution sample prepared for 2-DE (comb. elution fraction; 1/50 of the sample) were subjected to SDS-PAGE, as indicated. Gels were dried and exposed in a phosphorimager. An image of 32P-labeled proteins (**A**) and an image of silver-stained gel (**B**) are shown.

5. The membrane was washed 3 times with TBS-T for 15 min each wash.
6. The membrane was incubated with secondary antibody 30 min at room temperature on the rocking platform. The secondary antibody was freshly prepared prior to use with dilution 1:10000 in 5%BSA in TBS-T.
7. The membrane was washed 3 times with TBS-T for 15 min each wash.
8. The membrane was then incubated with Chemiluminescence Luminol Reagent (Santa Cruz, CA), and proteins were visualized by a suitable exposure with Kodak BioMax MR film. An example result is shown in **Fig. 1D**, **1E**, **1F**, and **1G**.

3.5.4. Separation of Eluted Fraction with 2-DE

1. Prepared samples (eluted proteins solubilized in rehydration solution from **step 3.3)** were subjected to isoelectric focusing using IPGDry strips with immobilized pH gradient, pH range 3–10, 18 cm, linear (GE Healthcare, Uppsala, Sweden) according to the following protocol: rehydration 10 h; 50 V, 3 h; 1000 V, 1 h; 8000 V, 10 h or to 50,000 Vh.
2. 2-D SDS–PAGE was performed in 12% polyacrylamide gels. The Ettan DALT*six* gel caster was used to cast the gels 5–13 h prior to 2-D electrophoresis. Gels were stored at room temperature prior to use.
3. The 2-D gels were run during the day (about 7 h) at constant power 5W per gel through the sealing agarose and 60 W in total through the acrylamide gel.
4. After the electrophoresis, gels were fixed in 10% acetic acid and 20% methanols for 10–12 h. Proteins were detected by silver staining, as described above.
5. To monitor efficiency of phosphoprotein enrichment the gels were exposed in a FujiX2000 PhosphorImager for 18 h.

◄

Fig. 1. (continued) Data obtained by densitometry of silver stainined gel and autoradiography were used to calculate an yield of phosphoproteins (Y_{pp}), concentration factor (C_{pp}) and level of phosphoproteins in the elution fraction (E_{pp}) as shown in panel **(C)**, where **A**, **C**, and **E** is densitometrical intensity of the input fraction, elution fraction 1 and elution fraction 2 correspondingly for silver stained gel; **B**, **D**, and **F** is densitometrical intensity of the input fraction, elution fraction 1 and elution fraction 2 correspondingly for autoradiography.

To monitor phosphorylated proteins, the gels were subjected to immunoblotting with antibodies specific to phosphotyrosine **(D)**, to phosphoserine **(E)**, or to phosphothreonine **(F)**. To monitor TGFb-induced phosphorylation of Smad2, MCF10A cells were treated or not with TGFb1 (5 ng/mL), as indicated, and phosphoproteins were enriched with Fe-IMAC procedure **(G)**. Collection of fractions and gel electrophoresis were performed as described in panels **A–E**. Immunoblotting was performed with antibodies specific to phosphorylated Smad2 (phosphorylated Smad2) or to total Smad2 (Smad2), as indicated. Lanes corresponding to the whole cell extract (input), flow-through (fl-thr), washing fractions (washes), and elution fractions (comb. elution fraction) are indicated. Combined elution fractions represent samples prepared for 2-DE (precipitated and solubilized elution fraction). Longer exposure with anti-Smad2 antibodies indicates presence of Smad2 in elution fractions. (Reproduced with permission from ***ref. 11***. Copyright Wiley-VCH Verlag GmbH & Co. KgaA.)

3.5.5. Estimation a Chromatography Efficiency Using 2-DE Gels

1. We performed 2-DE with eluted fractions, measured quantity-reflecting values of the silver-stained protein spots (quantitating volumes, as defined by the Image-Platinum software, GE Healthcare), and matched them with the radioactively labeled spots, which were detected in a phosphorimager.
2. Then, we compared the quantitating volume of proteins in silver-stained spots which corresponded to radioactively labeled proteins, to the total quantitating volume of silver-stained proteins in 2-D gels. We found that up to 90% of proteins of elution fractions, which were separated by 2-DE, were phosphorylated (**Fig. 2A** and **2B**).
3. The elution procedure allows the generation of good quality 2-D gel. In 2-D gels of proteins prepared with Fe-IMAC technique, we detected in average 500 ^{33}P- or ^{32}P-labeled proteins **(Fig. 2B)** and in average 700 silver-stained spots **(Fig. 2A)**. For comparison, we identified in average only 240 ^{32}P-labeled proteins in 2-D gels of whole cell lysate from MCF10A cells **(Fig. 2D)**. Silver staining revealed in average 1400 proteins spots in the same gel **(Fig. 2C)**.
4. To make sure that Fe-IMAC technique described here is compartible with mass-spectrometry, we randomly selected and identified more than 50 proteins from 2-D gels, which are shown in **Fig. 2**, using MALDI-TOF MS (**Table 1** shows identification of six proteins, as example). Searches of PubMed and prediction of phosphorylation sites with NetPhos showed that the identified proteins were reported as phosphorylated in cells and/or have a number of well-defined phosphorylation sites (**Table 1**). Together with ^{33}P- and ^{32}P-metabolic labeling and immunoblottings with anti-phospho amino acids antibodies (**Figs. 1** and **2**), this confirms efficient enrichment of phosphoproteins with Fe-IMAC.

4. Notes

1. It is important to use EDTA-free inhibitor cocktail. Presence of EDTA in lysis buffer may prevent phosphoprotein binding.
2. Be sure that you have a permission to work with radioactive material, otherwise contact your lab coordinator.
3. Do not use 1×PBS (phosphate buffered saline) for cell washing. Nonorganic phosphates may interfere with phosphoprotein binding by Fe-NTA resin and cause significant losses of phosphoproteins.
4. Clarified cell lysate has been incubated with Fe-NTA beads directly into the chromatography column. Be sure that column tip is closed prior to add cell lysate.
5. The tubes content must change color from dark to white otherwise extend incubation time to 10 min to make sure that all bound phosphoproteins were eluted. In average 0.3–0.4 mg of proteins were eluted when 2.5 mg of cellular proteins were taken as the input. The composition of the elution buffer was optimized for the most efficient recovery of proteins, and to

be compatible with further 2-DE separation. Elution with EDTA, phosphate salts, or with a buffer containing only SDS were found to be incompatible with 2-DE.

6. Shake the sample immediately after adding of methanol and acetic acid. The flakes of precipitated proteins should appear after incubation the samples on ice. Acetone or ethanol precipitation resulted in formation of

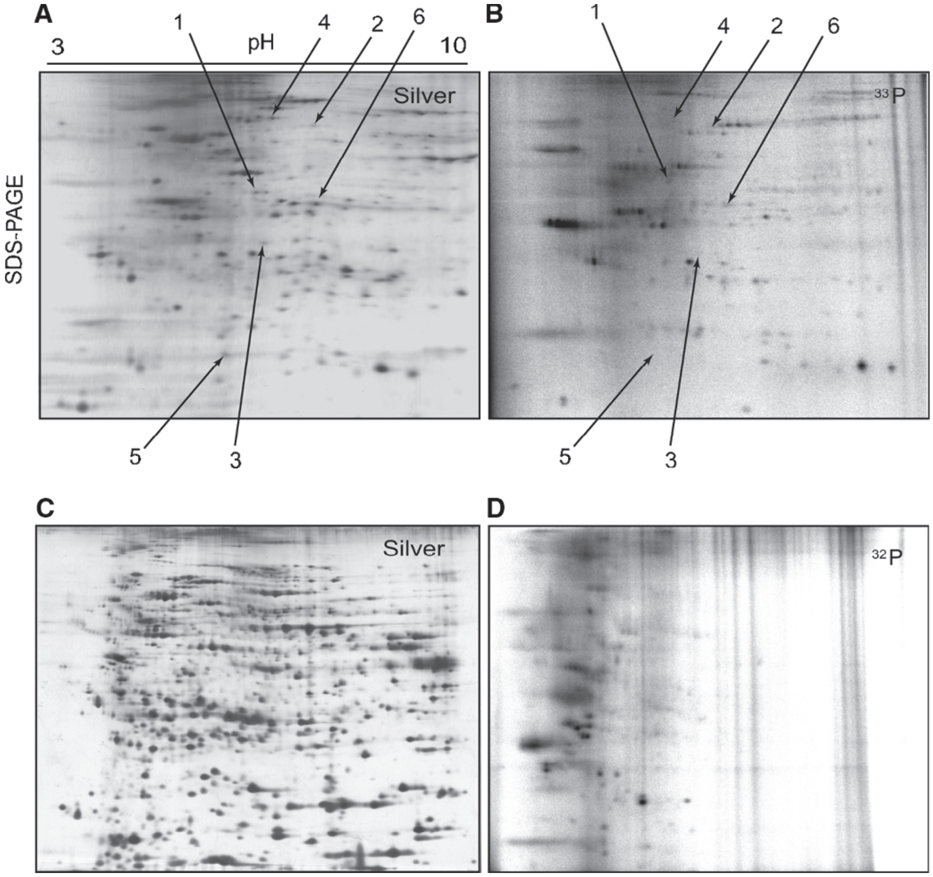

Fig. 2. Fe-IMAC protocol improves resolution of phosphorylated proteins in 2-DE. Images of 2-D gels obtained with samples of MCF10A cells prepared according to the Fe-IMAC protocol (**A**,**B**), prepared from the whole MCF10A cell extract (**C**,**D**), and prepared from chicken liver (**F**,**G**) are shown. Radioactive orthophosphate labeled proteins of MCF10A cells were detected after exposure gels in a phosphorimager, and images are shown in panels **B** and **D**. Images of the same gels after silver staining are shown in panels **A** and **C**. Arrows show migration positions of proteins listed in **Table 1**, as examples of identified proteins. Annotation of proteins (spot numbers) is as in **Table 1**.

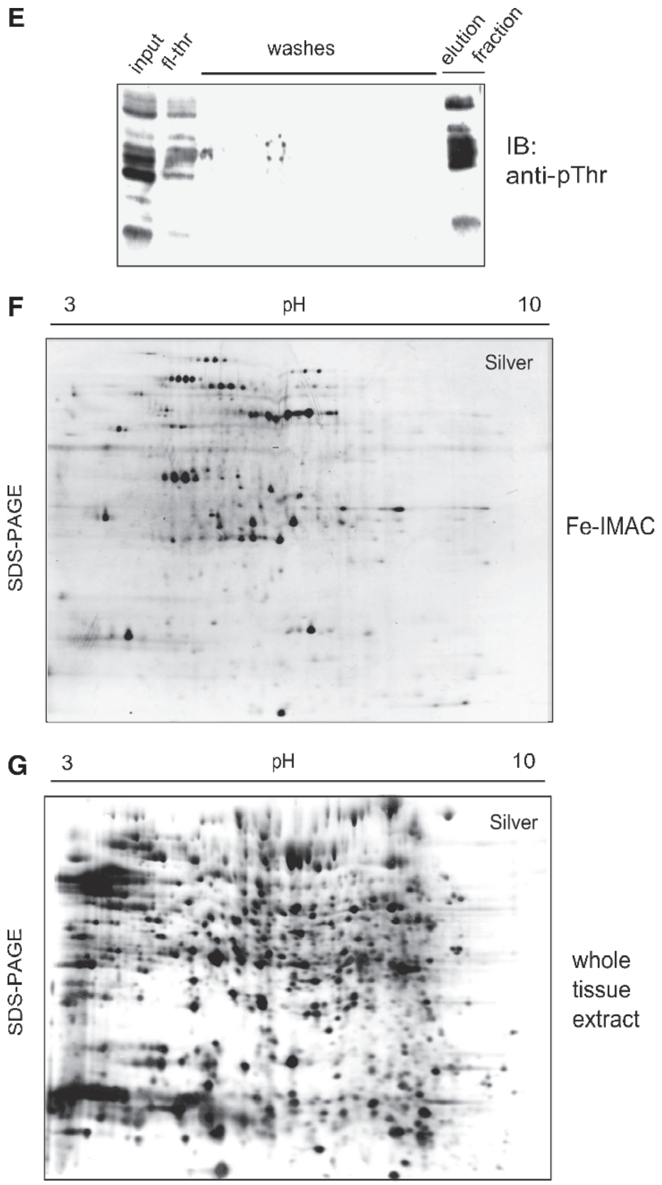
E
input
fl-thr
washes
elution fraction
IB:
anti-pThr
F
3
pH
10
Silver
SDS-PAGE
Fe-IMAC
G
3
pH
10
Silver
SDS-PAGE
whole
tissue
extract

Fig. 2. (continued) Fe-IMAC-based enrichment of phosphoproteins from chicken liver was monitored by 1-DE, followed by immunoblotting with antiphosphothreonine antibodies **(E)**. Images of silverstained 2-D gels obtained with Fe-IMAC sample **(F)**, and with whole liver extract **(G)** are shown. 2-DE was performed as described by Kanamoto et al. **(13)**. Directions of isoelectrofocusing (pH) and SDS-PAGE are indicated (directions in panel **A** are also for gels in panels **A–D**). (Reproduced with permission from ***ref. 11***. Copyright Wiley-VCH Verlag GmbH & Co. KgaA.)

Table 1

Spot[a]	Name of protein[b]	Accession number[b]	Probability[b]	*Z*-score[b]	Seq. cover.,%[b]	Experimental values		Theoretical values		Reference[c]
						p*I*	M_r	pI	M_r	
1	NudE	NP_110435	1.0e 1 000	2.43	14	5.6	42	5.2	38	[14]
2	Anaphase-promoting complex (APC, subunit 8)	T51168	1.0e 1 000	2.43	21	6.7	63	6.6	69	[15]
3	GTP-binding protein alpha q subunit	AAC50363.1	1.0e 1 000	2.43	27	5.7	26[d]	5.6	42[d]	[16]
4	HSP 70 (mortalin)	AAH30634.1	1.0e 1 000	2.43	22	5.8	75	6.0	74	[17]
5	Cytohesin1 B2-1	1bc9	1.0E 1 000	1.43	43	5.5	21	6.3	23	[18]
6	Grb14	AAH53559.1	1.0e 1 000	1.19	18	6.5	46[d]	9.0	61[d]	[19]

a) Annotation of protein spots is as indicated in Fig. 2.

b) Name of proteins, accession number, probability, *Z*-score, sequence coverage, and theoretical pI and M_r values were obtained searching NCBI database with ProFound search engine. Experimental values of pI and M_r were calculated from migration position of proteins in 2-D gels.

c) References to papers describing identified proteins as phosphoproteins.

d) Lower molecular masses of identified GTP-binding protein alpha *q* subunit and Grb14 may be due to the limited proteolysis of proteins.

precipitates which were not compatible with 2-DE. Dialysis of elution fraction did not eliminate completely the compounds interfering with 2-DE, or caused significant losses of phosphoproteins.

7. Be sure that no solution left in the tube prior to dissolve the pellet with rehydration solution.
8. Any bubbles trapped between the gel and the cellophane must be removed.
9. We monitored the phosphorylation of Smad2, which is a component of transforming growth factor-b (TGFb) signaling pathway, and which is phosphorylated at two C-terminal serine residues upon its activation (12). You can use anti-phospho- antibodies to any other proteins, which you can induce to be phosphorylated instead. It will provide you a good marker to check the phosphoprotein distribution between the chromatography fractions.

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Analyzing Protein Phosphorylation

John Colyer

1. Introduction

Protein phosphorylation is a ubiquitous modification used by eukaryotic cells to alter the function of enzymes, ion channels, and other proteins in response to extracellular stimuli, or mechanical or metabolic change within the cell. In many instances, phosphorylation results in a change in the catalytic activity of the phosphoprotein, which influences one particular aspect of cellular physiology, thereby allowing the cell to respond to the initiating stimulus. A number of different residues within a protein can be modified by phosphorylation. Serine, threonine, and tyrosine residues can be phosphorylated on the side chain hydroxyl group (*o*-phosphoamino acids), whereas others become phosphorylated on nitrogen atoms (*N*-phosphoamino acids, lysine, histidine, and arginine). The former group are involved in dynamic "regulatory" functions and have been studied extensively ***(1)***, whereas the latter group may perform both structural/catalytic roles and signaling functions, the study of which has occurred more recently ***(2)***. The disparity in our understanding of the role of *o*- and *N*-phosphoamino acids is in part a consequence of the acid lability of *N*-phosphoamino acids, which leads to their destruction during the analysis of many phosphorylation experiments.

In terms of the process of studying an individual phosphoprotein, a number of key issues can be identified. First, one must demonstrate that phosphorylation of the protein takes place; then define the number of sites within the primary sequence that can be phosphorylated and by which protein kinase; identify the individual residue(s) phosphorylated; the functional implication of phosphorylation of each site; and describe the use of each site of phosphorylation in vivo. This chapter aims to describe the conduct of an experiment performed to identify a protein as a phosphoprotein. In the case of oligomeric enzymes, it will identify the subunit(s) phosphorylated by a particular kinase. The determination of

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the stoichiometry of phosphorylation is also described, which provides the first information concerning the number of phosphorylation sites within a polypeptide. These procedures are most straightforward if one has access to the purified protein kinase of interest and the protein substrate. In the case of the kinase, this can be served in many instances by a number of commercial sources, but the approach may be limited by the availability of sufficient pure protein substrate. If this is the case, phosphorylation of a particular target as part of a complex mixture of proteins (e.g., whole-cell extract) can be performed. Under these conditions, identification of the protein of interest will require exploitation of a unique electrophoretic property of the protein ***(3)*** or require purification of the protein by immunoprecipitation or other comparable affinity-interaction means prior to electrophoresis. The identification of the protein as a phosphoprotein can thereby be achieved, although analysis of the stoichiometry of phosphorylation in this way is inadvisable. In each case, the experimental procedure has a common design: an in vitro phosphorylation reaction is followed by separation of the phosphoproteins by SDS-PAGE and subsequent identification by autoradiography. The incorporation of labeled phosphate can be determined by excising the phosphoprotein band from the dried gel, scintillation counting this gel piece, and converting ^{32}P cpm into molar terms from the knowledge of the specific activity of the initial ATP stock, and the amount of protein substrate analyzed.

2. Materials

1. Purified and partially purified multifunctional protein kinases can be obtained from several commercial sources. The availability of a number of enzymes is illustrated in **Table 1**. The list is not exhaustive, and inclusion in the table does not constitute endorsement of the product:

Table 1
Commercial Source of Multifunctional Protein Kinases

Kinase	Source
Protein kinase A	*a,c,d,e*
Protein kinase C	*a,b,c,d*
Calmodulin kinase II	*c*
Casein kinase II	*a,b,d*
CDC2 kinase	*d*
src kinase	*d*

[a] Boehringer Mannheim.
[b] Calbiochem-Novabiochem.
[c] Sigma Chemical Co.
[d] TCS Biologicals Ltd.

2. Phosphorylation buffer for the catalytic subunit of protein kinase A (c-PKA): 50 m*M* Histidine-KOH, pH 7.0, 5 m*M* $MgSO_4$, 5 m*M* NaF, 100 n*M* c-pKA, 100 µ*M* ATP.
   a. Histidine-KOH, pH 7.0 is prepared as a concentrated stock, 200 m*M* stored at 4°C for 1 mo, or −20°C for >12 mo. (Warm to 30°C for 30 min to dissolve histidine following storage at −20°C.)
   b. $MgSO_4$, EGTA, NaF: all prepared as 100-m*M* stock, stable at 4°C >12 mo.
   c. ATP, nonradioactive: 20- or 100-m*M* stock (pH corrected to 7.0 with KOH) stable at −20°C for >12 mo. Aliquot to avoid repeat freeze-thaw cycles.
   d. c-pKA, M_r 39,000 ***(4)***, sources **Table 1**: stable at −70°C for ~12 mo. Avoid dilute solutions—enzyme tends to aggregate and inactivate.
3. γ-^{32}P-ATP, ICN Pharmaceuticals: dispense into small aliquots and store −20°C. Avoid freeze to thaw cycles. $T_{1/2}$ ~14 d; discard 1 mo after reference date.
4. SDS-PAGE sample buffer, (double-strength): 125 m*M* Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue, stable at −20°C >12 mo. Aliquot and avoid freeze-thaw cycles.
5. Filter paper: Whatman 3MM.
6. X-ray film, X-ray cassettes, intensifying screens, developer and fixative: X-Ograph Ltd.; developer and fixative are stored at room temperature, are reusable, and are stable for approx 2 wk.
7. Scintillation fluid: Emulsifier-safe, Packard.

3. Method

3.1. Phosphorylation Reaction Using Purified Protein Substrates

1. In a designated radioactive area with appropriate acrylic screening prepare a stock of radioactive ATP. The addition of 50 µCi [γ-^{32}P]-ATP to a 0.5-mL solution of 1 m*M* ATP will produce a suitable experimental ATP stock of 220 cpm/pmol (*see* **Note 1**). Warm to 37°C.
2. Incubate the purified protein of interest (0.1–1.0 mg/mL) in the phosphorylation assay medium lacking ATP. Allow the sample to warm to 37°C for 2 min (*see* **Note 2**). A number of control samples should be set up in parallel. One control should contain target protein, but no exogenous kinase, and another, kinase but no target protein.
3. Start the phosphorylation reaction by the addition of ATP, containing [γ-^{32}P]-ATP (as defined in **step 1, Subheading 3**.). Cap the tube and vortex briefly. Follow the phosphorylation as a function of time by removing aliquots of the reaction at specific points in time, every 20 s for the first minute, and then at 60-s intervals for the next 4 min.
4. Terminate the reaction by mixing the sample with an equal volume of double-strength SDS-PAGE sample buffer at room temperature. Dispense the phosphorylation sample into an Eppendorf tube containing an equal volume of double-strength sample buffer, cap the tube, and vortex briefly. Store these samples at room temperature, behind appropriate screens, until all samples have been collected.

5. Incubate all samples for 30 min at 37°C. Perform SDS-PAGE in a gel of suitable acrylamide composition, loading a minimum of 5 µg pure target protein/lane (details of electrophoresis in Chapter 21). Allow the dye front to migrate off the bottom of the gel (depositing most of the radioactivity into the electrode buffer), stain with Coomassie brilliant blue, and destain the gel (*see* Chapter 21).
6. Mount the gel on filter paper and cover with clingfilm. The filter paper should be wet with unused destain solution prior to contact with the SDS-PAGE gel. Lower the gel onto the wet filter paper slowly, and flatten to remove air bubbles. Cover with clingfilm, and dry using a vacuum-assisted gel drier for 2 h at 90°C.
7. Once dry, an X-ray film should be placed in contact with the gel for a protracted period to image the location of phosphoproteins. **This procedure must be performed in the dark**, although light emitted by dark room safety lamps is permitted. An X-ray film is first exposed to a conditioning flash of light from a flash gun. Hold a single piece of X-ray film and the flash gun 75 cm apart. Set the flash gun to the minimum power output, and discharge a single flash directly onto the film (*see* **Note 3**). Place the film on top of a clean intensifying screen within an X-ray cassette. Take the dried SDS-PAGE gel, still sandwiched between filter paper and clingfilm, and place it gel side down onto the X-ray film. Do not allow the gel to move once in contact with the film, and use adhesive tape to secure the contact. With a permanent marker pen, draw distinctive markings from the filter paper backing of the gel onto the X-ray film to facilitate orientation of film and gel once autoradiography is complete (*see* **Note 4**). Close the X-ray cassette, label the cassette with experimental details, including the current date and time, and store at −70°C.
8. After 16 h of exposure, the autoradiograph can be developed. Remove the cassette from the −70°C freezer, and allow at least 30 min for it to thaw. Once in the dark room, with safety light illumination only, the cassette should be opened, and SDS-PAGE gel removed from the X-ray film. The film should be placed in 2 L developer and agitated for 4 min at room temperature, in the dark. Using plastic forceps, the film is removed from developer, rinsed in water, and then agitated for a further 90 s in 2 L of fixative (room temperature, dark). At the end of 90 s, the autoradiograph is no longer light-sensitive, and normal lighting can be resumed. Wash the autoradiograph extensively, for 10 min in a constant flow of water, and allow to air-dry.
9. Identification of phosphorylated polypeptides can be performed by superimposition of autoradiograph on the SDS-PAGE gel (*see* **Note 5**). A uniform, almost transparent background should be achieved, with phosphorylated proteins identified as black bands on the autoradiograph of variable intensities (depending on the level of phosphorylation), but regular width and shape (*see* **Note 6**). Exposure times can be altered in the light of results obtained, and repeated autoradiographs of various durations performed on a single gel (*see* **Note 7**).

3.2. Phosphorylation of Components in Complex Protein Mixtures

1. The phosphorylation experiment is performed largely as detailed in **Subheading 3.1**. with the following modifications. A protein concentration of 1–5 mg/mL is recommended supplemented with 100 n*M* purified c-pKA and 10 µ*M* adenosine

cyclic 3,5-monophosphate, and with 1 µ*M* microcystin-LR for additional Ser/Thr phosphatase control.

2. Perform a phosphorylation time-course experiment and process as described in **Subheading 3.1**. If the identity of a particular protein cannot be gauged from a peculiar electrophoretic feature (e.g., dissociation of oligomer to monomer on boiling; ***3***), then an affinity purification step must be introduced prior to electrophoresis.
3. In this case, phosphorylation will be terminated by placing the sample on ice. Immunoprecipitation of the protein of interest should be performed, taking care to solubilize membrane proteins effectively if they are of interest. Immunoprecipitates should be processed as described in **Subheading 3.1., step 5** onward (*see* **Note 8**).

3.3. Determination of Phosphorylation Stoichiometry

1. The specific activity of the ATP (cpm/pmol) needs to be determined empirically. At the time of the phosphorylation experiment, dilute a sample of the experimental ATP (1 m*M* containing 100 µCi/mL [γ-^{32}P]-ATP, as described in **Subheading 3.1., step 1**) to 1 µ*M* by serial dilution in water. Dispense triplicate 10-µL aliquots of the 1-µ*M* ATP (10 pmol) into separate scintillation vials, and add 4.6 mL scintillation fluid to each. Cap and label 10 pmol ATP (*see* **Note 9**).
2. Perform **steps 1–9** of **Subheading 3.1**. To excise the phosphorylated protein bands from the dry SDS-PAGE gel, identify the location by superimposition of gel and autoradiograph. With a marker pen, outline the autoradiographic limits of the phosphoprotein on the gel. Overlay again to confirm the accuracy of demarkation. Mark similar-sized areas of gel that do not contain phosphoproteins to determine background [^{32}P]. Excise these gel pieces with scissors, remove the clingfilm, and place the acrylamide piece and filter paper support in a scintillation vial. Add 4.6 mL scintillation fluid, and cap the vial.
3. Scintillation count each vial for 5 min or longer, using a program defined for ^{32}P radionucleotides. Minimal quenching of ^{32}P occurs under these conditions.
4. To calculate the pmol phosphate incorporated/µg protein, subtract the background radioactivity (cpm) from experimental data (cpm) to obtain phosphate incorporation into the protein sample (in units of cpm). Convert this to pmol incorporation/µg using the formula:

$$\text{Phosphorylation (pmol/}\mu\text{g protein)} = [\text{protein phosphorylation (cpm)/SA of ATP (cpm/pmol)} \times \mu\text{g protein}] \quad (1)$$

With a knowledge of the molecular weight of the polypeptide, a molar phosphorylation stoichiometry can be calculated from these data using the formula:

$$\text{Phosphorylation (mol/mol protein)} = [\text{phosphorylation (pmol/}\mu\text{g protein)} \times \text{molecular weight}/10^6] \quad (2)$$

5. Experimental conditions that result in maximal protein phosphorylation will have to be optimized. Parameters worth considering include alteration of the pH of the reaction, extension of the time-course of phosphorylation (up to several hours), addition of extra protein kinase during the phosphorylation process, and addition of extra ATP throughout the time-course.

4. Notes

1. The specific activity of ATP must be tailored to the experiment intended. Phosphorylation and autoradiography of proteins require ≥200 cpm/pmol, studies that require analysis beyond this point (e.g., phosphoamino acid identification) require ~2000 cpm/pmol, while phosphorylation of peptides requires ~20–50 cpm/pmol.
2. The stability of proteins at low concentration is sometimes an issue; inclusion of an irrelevant protein, but not a phosphoprotein (e.g., bovine serum albumin) at 1 mg/mL (final) is recommended. The phosphorylation example used to illustrate this method (c-pKA) displays catalytic activity in the absence of signaling molecules. In other instances this is not so. Therefore, relevant activators should be included as dictated by the kinase (e.g., Ca^{2+}, calmodulin, acidic phospholipids, and so forth).
3. Film developed at this stage will exhibit very slight discoloration compared to unexposed film.
4. Phosphorescent labels (Sigma-Techware) can be used to label an SDS-PAGE gel prior to autoradiography. It will also facilitate superimposition of gel and autoradiograph. These can also highlight the position of mol-wt markers, an image of which will be captured on the X-ray film.
5. Protein kinases invariably autophosphorylate. This can be identified clearly in control samples lacking phosphorylation target (**Subheading 3.1., step 2**).
6. Autoradiographs sometimes have a high background signal. Uniform black coloration over the whole film, extending beyond the area exposed to the gel is indicative of illumination of the X-ray film. Discoloration of part of the film is indicative of light entering the X-ray cassette. Examine the cassette carefully, particularly the corners that are prone to damage by rough handling.
7. Phosphoimage technology represents an alternative to autoradiography, it has the advantage of collecting the image quickly. Exposure times are reduced by an order of magnitude. However, in my experience, this benefit is at the expense of the quality of the image, which is granular.
8. The time required for immunoprecipitation or similar procedure should be kept to a minimum to limit the dephosphorylation of proteins by endogenous phosphatase enzymes. A cocktail of phosphatase inhibitors should be included for the same reason.

9. The hydrolysis of ATP to ADP and Pi occurs at a low rate in the absence of any enzyme. The extent of hydrolysis of [γ-^{32}P]-ATP can affect the determination of phosphorylation stoichiometry, since it will result in the overestimation of the specific activity of ATP if a correction is not made. Quantification of the purity of ATP is quoted in the product specification from suppliers. Only fresh [γ-^{32}P]-ATP should be used in these procedures or the degree of hydrolysis confirmed by thin-layer chromatography ***(6)***.

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Mass Spectrometric Analysis of Protein Phosphorylation

Stefan Gander, Alessio Cremonesi, Johana Chicher, Suzette Moes, and Paul Jenö

1. Introduction

Phosphorylation is one the most frequently occurring posttranslational modifications in proteins, playing an essential role in transferring signals from the outside to the inside of a cell and in regulating many diverse cellular processes such as growth, metabolism, proliferation, motility and differentiation. It is estimated that up to one-third of all proteins in a typical mammalian cell are phosphorylated ***(1)***. Phosphorylation is carried out by a vast group of protein kinases which are thought to constitute 3% of the entire eukaryotic genome ***(1–3)***. To decipher the recognition signal of protein kinases and protein phosphatases acting on a given molecular target, and to understand how the activity of the target protein is regulated by phosphorylation, it is important to define the sites and the extent of phosphorylation at each specific site.

Earlier techniques of phosphoprotein analysis involves in vivo or in vitro labeling with [^{32}P]-phosphate ***(4–8)***. The radiolabelled protein is subsequently digested with a suitable protease and radiolabelled peptides are separated either by high-performance liquid chromatography (HPLC) ***(7,8)*** or by two-dimensional phosphopeptide mapping ***(4–8)***. The site of phosphorylation is then determined by solid-phase Edman sequencing ***(6)***. Identification of phosphorylation sites by Edman degradation of [^{32}P]-labelled proteins has been almost completely replaced by mass spectrometric techniques mainly because of speed, versatility and the availability of powerful computational tools ***(9–12)***. MS-based techniques have been steadily improved in the past so that the global analysis of protein phosphorylation, its dynamics and the elucidation of substrates phosphorylated

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by protein kinases have become possible ***(13,14)***. These studies have provided remarkable insights into signaling cascades and their organization.

The basic elements of mass spectrometric phosphoprotein analysis consist of proteolytic digestion of the protein(s) of interest followed by measuring the masses of the resulting peptides. Individual phosphopeptides can be detected by a variety of techniques such as MALDI-mass mapping and phosphatase treatment ***(15)***, neutral loss scanning ***(16)***, and precursor ion scanning ***(17)***. The phosphorylation site in a given phosphopeptide can be identified by subjecting the peptide to fragmentation in the collision cell of the mass spectrometer. From the mass difference between the fragment ions, the sequence can be "read" and the site of phosphorylation can be identified when the mass difference is either 167, 181, or 243 Da, corresponding to the residue masses of P-Ser, P-Thr, or P-Tyr. Powerful search programs such as SEQUEST and MASCOT ***(18,19)*** exist to identify both, the phosphoprotein and the site at which the phosphate resides.

Phosphorylation at Ser, Thr, and Tyr residues may modulate the activity of a given protein. Thus, the determination of the extent to which a protein becomes phosphorylated is important for the understanding of the dynamics of how a protein responds to a particular stimulus. Recently, mass-spectrometry based techniques for relative quantification of protein phosphorylation have been developed, using either stable-isotope tagged amine-reactive labels (iTRAQ) ***(20)*** or stable isotope labeling by amino acids in cell culture (SILAC) ***(21)***. Alternatively, chemical ***(22)*** or enzymatic approaches ***(23)*** can be used to quantitate relative changes in phosphorylation.

One major obstacle in phosphoprotein analysis is the low relative level of phosphorylation, in particular tyrosine phosphorylation. To access phosphorylation events, enrichment strategies have been developed that allow selective isolation of serine/threonine phosphorylation by immobilized metal affinity chromatography (IMAC) ***(24)*** or tyrosine phosphorylation by immunoprecipitation with antiphosphotyrosine antibodies ***(25)***.

In this chapter, we will describe the basic steps of mass spectrometric phosphopeptide mapping, namely enzymatic digestion of phosphoproteins, reverse-phase high-performance liquid chromatography of peptides with mass spectral analysis (LC/MS), and determination of the site of phosphorylation by tandem mass spectrometry (MS/MS).

2. Materials

2.1. Equipment

1. HPLC system: For LC/MS coupling, a Rheos 2200 pump (Flux Instruments, Basel, Switzerland) was operated at a flow of 150 ml/min. A precolumn split was used to reduce the flow to approximately 500 nl/min.

2. Mass Spectrometers: LTQ Orbitrap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a Finnigan Nanospray Probe (Thermo Electron, San Jose, CA) and Bruker Reflex III MALDI-TOF instrument (Bruker Daltonics GmbH, Switzerland).

2.2. Materials and Reagents

1. Fused silica capillaries (100 μm i.d., 280 μm o.d.) and PEEK sleeves (300 μm i.d.) (LC Packings, Dionex Switzerland AG, Olten, Switzerland). F120 PEEK Fingertight fittings (Upchurch Scientific, ERCATECH AG, Berne, Switzerland).
2. C_{18} capillary columns for LC/MS: 300SB C-18 columns, 0.3×50 mm (Agilent Technologies, Basel, Switzerland). C_{18} ZipTips, P10 size, were from Millipore (Bedford, MA). PHOS-Select Iron affinity Gel was purchased from SIGMA-ALDRICH (Buchs, Switzerland).
3. Chemicals: TFA (Applied Biosystems, Rotkreuz, Switzerland) and acetonitrile (Lab-Scan, MACHEREY-NAGEL AG, Oensingen, Switzerland). DTT and ten times concentrated dephosphorylation buffer (0.5 *M* Tris-HCl, pH 8.5, 1 m*M* EDTA) (Roche Diagnostics, Mannheim, Germany). Iodoacetamide (Fluka Chemie AG, Buchs, Switzerland).
4. Enzymes: Proteases were obtained from the following suppliers: modified trypsin: (Promega, Madison, WI), endoproteinase LysC (Achromobacter protease) (Wako Pure Chemical Industries, Osaka, Japan), endoproteinase GluC, sequencing grade, and calf intestinal alkaline phosphatase (1000 U/ml) (Roche Diagnostics, Mannheim, Germany).
5. Isotopically labeled $H_2{}^{18}O$, >95% pure (CAMPRO Scientific GmbH, Berlin, Germany). α-cyano-4-hydroxycinnamic acid for MALDI-TOF (Sigma-Aldrich, Buchs, Switzerland).

3. Methods

3.1. Enzymatic Digestions of Phosphoproteins

To efficiently trace sites of phosphorylation in proteins, it is important to obtain an essentially complete proteolysis of the protein of interest (*see* **Note 1**). This is best achieved if the protein is fully denatured by reduction and alkylation with DTT and iodoacetamide. For reduction, the protein is dissolved in 10 μl 100 m*M* Tris-HCl, pH 8.0, 8 M urea (freshly prepared), 1.5 μl 75 m*M* DTT dissolved in water) is added and the protein is reduced for 1–2 h at 37°C. Alkylation is done by adding 1 μl of 625 m*M* iodoacetamide and incubating the protein for 15 min at room temperature.

To obtain an as complete a digest as possible, digestion with a combination of proteases is preferable. After reduction and alkylation the protein is first digested with the endoproteinase LysC. The residual iodoacetamide and urea (approximately 6 *M*) do not appreciably inhibit the enzymatic activity. The enzyme to substrate ratio is kept between 1:50 and 1:20 (w/w). Incubation with endoproteinase LysC is carried out at 37°C for 1 h. The reaction is stopped by the addition of 10% TFA to a final concentration of 0.5% TFA. For subsequent trypsin digestion, the urea concentration has to be lowered to 2 M by dilution with 100 mM Tris-HCl, pH 8.0, whereas endoproteinase GluC is severely inhibited even by 2 M urea. When using this enzyme, it is best to remove urea completely. This can be achieved by reverse-phase chromatography. Digestion with trypsin or endoproteinase GluC is carried out at an enzyme to substrate ratio of 1:50 to 1:20 (w/w) for 18 h at 37°C.

3.2. Phosphorylation Analyzed by Liquid Chromatography-Mass Spectrometry

To find phosphopeptides in protein digests, reverse-phase chromatography in MS-compatible solvents such as formic acid or acetic acid is directly coupled to the mass spectrometer. The eluting peptides are measured in a data-dependent mode. First, full-range mass spectra are analyzed in real time, and then ions are collected for fragmentation to generate sequence-specific ions for data bank searching. For phosphopeptide detection we rely in most cases on neutral loss scanning. The neutral loss is the result of a gas-phase b-elimination reaction of phosphoric acid (–98 Da) from phosphoserine and phosphothreonine. Peptides phosphorylated on serines and threonines can be found by searching the LC/MS data for those peptides that had lost either 49, 32.6, or 24.5 Da for doubly, triply, or quadruply charged peptides. P-Tyr containing peptides do not tend to lose phosphoric acid and escape detection by neutral loss scanning. Individual MS/MS spectra are then compared against the known protein sequence using Turbo-Sequest software *(26)*. **Fig. 1A** shows the LC/MS analysis of a tryptic digest of the yeast ribosomal protein RPL24A (systematic yeast gene annotation is YGL031C). In particular, we were interested in the peptide GDSKIFR surrounding Ser21 that had been reported to be phosphorylated in vivo *(21)*. The phosphorylated doubly charged peptide eluted at 30.49 min from the reverse phase column **(Fig. 1B)**. Searching the LC/MS data for the neutral loss of phosphoric acid for doubly charged peptides (49 Da) revealed a strong candidate peptide whose elution coincides with the predicted phospho-GDS*KIFR peptide **(Fig. 1C)**. Fragmentation of the phosphopeptide was triggered at scan 5208 (30.43 min). The primary fragmentation product is an intense ion (402.67 Da) 49 Da less than the precursor ion **(Fig. 2A)**. While the doubly charged ion of 451.7 Da matches exactly the singly phosphorylated peptide GDSKIFR, positive identification by data bank searching with the TurboSEQUEST software was not possible due to the few fragment ions present in the spectrum. This is typical for phosphopeptides that tend to predominantly lose phosphoric acid via b-elimination and therefore suppress the formation of informative fragment ions. Therefore, the 402.67 ion that underwent neutral loss of phosphoric acid was brought to fragmentation in the subsequent scan. This resulted in a richer fragmentation spectrum from which the phosphopeptide and

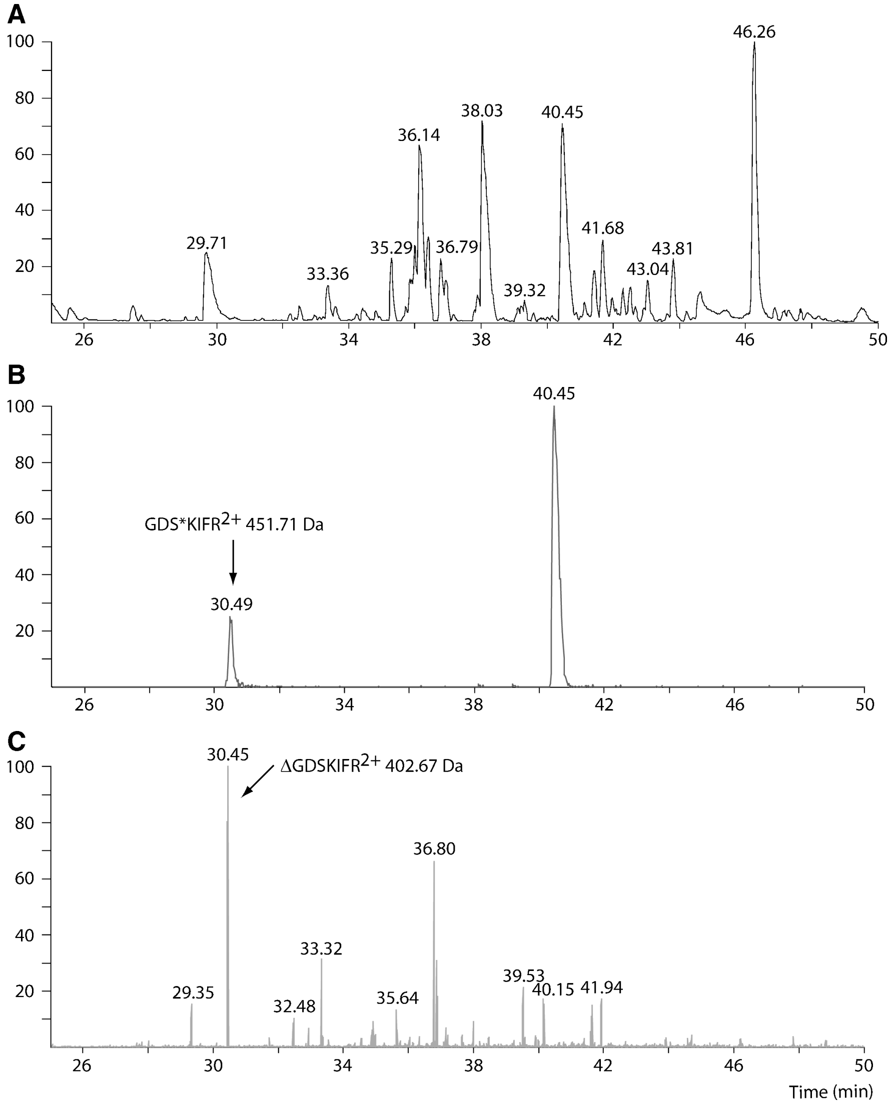

Fig. 1. **(A)** Ion chromatogram of an endoproteinase LysC/tryptic digest of yeast ribosomal proteins eluting from a capillary column into the mass spectrometer. **(B)** Selected ion tracing for the 451.71 Da ion that corresponds to the singly phosphorylated GDS*KIFR from ribosomal protein RPL24A (YGL031C). **(C)** Neutral loss scanning experiment showing the ion tracing for doubly charged peptides that underwent loss of phosphoric acid (49 Da). The asterisk indicates phosphorylation, and D marks the peptide that had lost phosphoric acid during collisional activation.

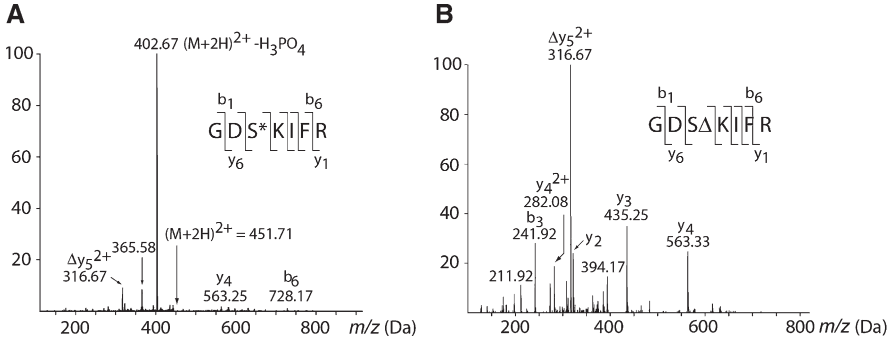

Fig. 2. Fragmentation spectrum of the doubly charged GDS*KIFR phosphopeptide. **(A)** The 451.71 Da precursor ion caused a prominent loss of phosphoric acid (402.67 Da) with little fragmentation of the peptide backbone. **(B)** Abundant peptide fragmentation occurs upon further fragmentation of the 402.67 Da neutral loss fragment ion (MS/MS/MS). The asterisk indicates phosphorylation, while D marks the serine that had lost phosphoric acid during collisional activation.

the site of phosphorylation could be unambiguously identified **(Fig. 2B)**. The "phosphate-specific" MS scan takes advantage of the neutral loss of phosphoric acid from phosphopeptides in a fully automated fashion (see notes). Once all the data have been collected, data bank search is initiated with the following settings: variable modification of Ser, Thr, and Tyr set to 80 Da (the mass of a phosphate) and 57 Da static modification for Cys (alkylation by iodoacetamide).

3.3. Stoichiometry of Phosphorylation

Stoichiometries of phosphorylation by mass spectrometry can be quantified by dividing the sum of the peak areas of all intensities of a given phosphopeptide (all charge states must be included) by the sum of all intensities of the phosphorylated and its nonphosphorylated forms. However, this often results in underestimating the phosphorylation stoichiometriy, as phosphopeptides tend to ionize with lower efficiency than their corresponding unphosphorylated peptides *(27)*. Quantitation in site-specific phosphorylation has been achieved by the use of stable isotope labeling *(28)* and most recently, by iTRAQ *(29)*. For the quantification of relative changes in the extent of phosphorylation that occur upon drug treatment of yeast cells we rely on a simple method that involves the labeling of individual pools of differentially phosphorylated proteins by enzymatic digestion in isotopically labeled water, affinity selection of phosphopeptides followed by MALDI time-of-flight mass spectrometry *(23)*. The method consists of three steps: (i) enzymatic digestion of a protein in $H_2^{16}O$ or isotopically enriched $H_2^{18}O$ to label individual pools of differentially phosphorylated proteins; (ii) affinity selection of phosphopeptides from combined digests by immobilized metal-affinity chromatography; and (iii) dephosphorylation with alkaline phosphatase to allow for quantitation of changes of phosphorylation by MALDI-TOF.

We have successfully used ^{18}O labeling to trace relative changes in phosphorylation of the yeast protein kinase Npr1 (systematic yeast gene name YNL183C). Upon treatment of yeast cells with rapamycin or following nitrogen starvation, Npr1 becomes activated by dephosphorylation to support sorting of the general amino acid permease Gap1 to the plasma membrane ***(30)***. We mapped the global changes of phosphorylation in Npr1 following rapamycin treatment. Particularly sensitive phosphorylation sites to rapamycin treatment are found on Ser353 and Ser356. The 1227.4 Da MALDI-TOF signal corresponds to the doubly phosphorylated peptide SQHSSIGDLKR (comprising residues 353–363) from untreated cells while the 1231.4 Da signal derives from the same phosphopeptide of Npr1 that had been obtained from rapamycin-treated yeast cells **(Fig. 3A)**. The same Npr1 phosphopeptide was isolated from a genomically deleted Sit4 mutant. In this case, no decrease in the extent of phosphorylation of

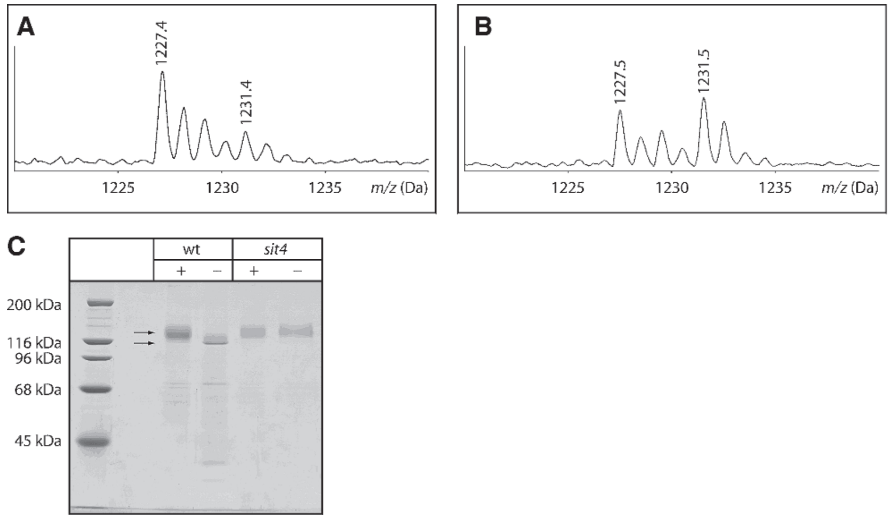

Fig. 3. (A) MALDI-TOF spectra of the doubly phosphorylated peptide (S*QHS*SIGDLKR) encompassing residues 353–363 of the yeast protein kinase Npr1 (YNL183C) that had been isolated from untreated (1227.4 Da) or from rapamycin-treated (1231.4 Da) cells. The 1231.4 Da signal indicates an approximately four-fold dephosphorylation. (B) The same phosphopeptide was obtained from a mutant in which the sit4 phosphatase responsible for Npr1 dephosphorylation was deleted. The 1231.5 Da signal indicates that no dephosphorylation occurred at these sites. (C) Coomassie-blue stained SDS gel of GST-Npr1 from wild-type cells (lane 1 and 2) or sit4 mutant cells, either untreated (−) or treated (+) with 200 nM rapamycin for 15 min. The arrows indicate phosphorylated (upper) and dephosphorylated (lower) GST-Npr1. (Modified from ***ref. 23***.)

the SQHSSIGDLKR phosphopeptide was detectable due to the lack of the Sit4 phosphatase responsible for dephosphorylation of Npr1 **(Fig. 3B)**.

3.4. Procedure for ^{18}O Labeling of Peptides

1. Dry 20 µl of reduced and carboxamidomethylated phosphoprotein (in 100 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 0.2 µg/µl protein concentration) in a SpeedVac.
2. Dissolve one pool of phosphoprotein in 20 µl $H_2{}^{16}O$ (for example, phosphoprotein derived from non-treated or wild type cells) and the other pool (for example, phosphoprotein derived from treated or mutant cells) in 20 µl isotopically enriched $H_2{}^{18}O$.
3. Digest the two protein pools separately with trypsin at an enzyme/substrate ratio of 1:25 at 37°C for 2 h. During digestion in $H_2{}^{18}O$, two atoms of ^{18}O will be incorporated into every newly generated peptide C-terminus.
4. Stop the digestion by adding TFA to a final concentration of 0.1%. Mix the digest from normal and from $H_2{}^{18}O$ water and desalt them by adsorbing the peptides onto a C_{18} ZipTip. The peptides are washed with 0.1% TFA and desorbed with 5 µl 80% acetonitrile containing 0.1%TFA.
5. Select phosphopeptides by adding approximately 2 ml of a 50% suspension of PHOS-Select IMAC beads (in 0.1% acetic acid/30% acetonitrile) to the desalted peptide pool. Incubate the peptide-IMAC suspension in an Eppendorf Thermoshaker (set to 1,200 rpm) at room temperature for 1 h.
6. Wash the beads three times with 25 ml 0.1% acetic acid/30% acetonitrile.
7. Release the bound phosphopeptides with 10 ml 100 m*M* Tris-HCl, pH 8.0 containing 0.5 units of alkaline phosphatase by shaking the suspension at 37°C for 15 min.
8. For MALDI-TOF analysis desalt the peptides with ZipTips as above, except that peptide elution is done with 80% acetonitrile, 0.1% TFA, containing 1 mg/ml a-cyano-4-hydroxycinnamic acid.

4. Notes

1. For routine phosphopeptide mapping, we usually digest the proteins first with the endoproteinase LysC followed by a second digestion with trypsin. This digestion regime generates peptides with at least two positive charges on each peptide for efficient ionization. The C-terminal lysine or arginine favours fragmentation in the collision cell, which leads to a high sequence coverage, a prerequisite for unambiguous localization of the site of phosphorylation. Also, since the endoproteinase LysC is active in 6 *M* urea, highly insoluble phosphoproteins can be efficiently digested. Subsequent trypsin digestion also is possible after lowering the urea concentration to 2 *M*.
2. One major obstacle in phosphopeptide analysis is that they are often too hydrophilic to bind to reverse-phase supports used for LC/MS separation.

A simple solution to this problem has been suggested by combining graphite powder for retaining phosphopeptides that are otherwise lost in the flow through ***(31)***.

3. In cases where phosphorylation is suspected but could not be proven due to poor fragmentation, we dephosphorylate the digest with alkaline phosphatase and repeat the analysis. If the candidate peptide is phosphorylated, dephosphorylation leads to a reduction of its mass by 80 Da (or multiples thereof in the case of multiple phosphorylation). Enzymatic dephosphorylation with calf-intestinal alkaline phosphatase (CIP) can be directly carried out in the digestion mix, since CIP is completely resistant to proteolysis by enzymes such as trypsin, endoproteinase LysC or -GluC, although urea tends to inactivate the enzyme. For protein digests in the 1–500 picomole range, 1 U of CIP was found to be sufficient to lead to complete dephosphorylation of phosphopeptides.
4. For phosphopeptide identification of LC/MS/MS data we rely on the SEQUEST software (Thermo Finnigan, San Jose, CA). The routine search settings are as follows: Delta CN for peptides is 0.1; Xcorr versus charge state is set to 1.50 for singly, 2.0 for doubly, and 2.5 for triply charged peptides, respectively; minimal protein probability is 0.01.
5. In principle, phosphorylation stoichiometries can be quantified by comparing the integrated peak intensities of a phosphopeptide and its nonphosphorylated counterpart. In doing so, it is assumed that both forms of the peptide ionize with equal efficiencies. This estimation of stoichiometries is in many cases valid for singly and doubly phosphorylated peptides. However, with increasing clustering of phosphorylation sites we have found that extensively phosphorylated peptides tend to become poorly ionized. In such cases, we prefer $H_2{}^{18}O$-labelling and phosphatase treatment. This allows estimation of relative changes in the extent of phosphorylation because an isotopically labelled pair of peptides with equal ionization prope ties is generated. Also, for MALDI-TOF experiments, neutral loss of phosphate does not occur, while the relative extent of phosphorylation is preserved. Absolute quantitation of the extent of phosphorylation is possible with the recently introduced AQUA technology ***(32)***.
6. The search parameters for the identification vary, of course, with the software used for protein identification. With the SEQUEST protein/peptide identification, we set the search parameters variable for Ser/Thr/Tyr phosphorylation (+80 Da) and variable Ser/Thr modification to −18 Da for those peptides that underwent loss of phosphoric acid and that had been fragmented in an MS3 scan.

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Protein Microarrays for Phosphorylation Studies

Birgit Kersten and Tanja Feilner

1. Introduction

Posttranslational modification of proteins by phosphorylation is the most abundant type of cellular regulation which affects essentially every intracellular process of eukaryotes. Phosphorylation of a protein can cause changes in its structure, stability, enzymatic activity, the ability to interact with other molecules, or its subcellular localization. Protein kinases are catalyzing the reversible phosphorylation of protein substrates at serine, threonine or tyrosine residues. In Arabidopsis, e.g., serin/threonine kinases represent about 4% of the proteome ***(1)***, but their biological function is not well understood. Therefore, high-throughput proteomics methods ***(2,3)*** for global analysis of protein kinase function are needed to identify downstream substrates. Various techniques for determining consensus phosphorylation site sequences, including peptide libraries ***(4)*** and peptide arrays ***(5,6)*** led to the identification of kinase substrates. Furthermore, a solid-phase phosphorylation screening of λ phage c-DNA expression libraries ***(7,8)*** as well as different protein-protein-interaction screening methods, such as overlay methods ***(9–11)*** and the yeast two hybrid system ***(12–14)*** have been applied in this respect. More recently protein kinases were engineered to accept unnatural adenosine triphosphate (cyclopentyl ATP) analogues and have been used to identify specific substrates ***(15–17)***. Furthermore, preliminary studies demonstrated the suitability of protein microarrays to study phosphorylation by kinases ***(18,19)***. To elucidate the sites of phosphorylation, antibodies against phosphorylated protein epitopes ***(6)*** may be used for detection on protein microarrays. Furthermore, peptide arrays ***(5,6)*** or MS-based methods ***(20,21)*** may be applied in this respect.

We here describe a protein microarray-based method for the phosphorylation screening of proteins and the identification of potential kinase substrates in a high-throughput manner. We successfully used this screening tool to

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identify novel targets for the barley casein kinase 2α (CK2α) ***(22)*** as well as for different *Arabidopsis thaliana* mitogen activated kinases (MAP kinases) ***(23)***. Our method utilizes plant protein microarrays which have been generated as described in detail previously ***(22, 24)***. Then microarrays have to be incubated with the soluble and active kinase in the presence of radioactive [γ^{33}-P]ATP. Radioactive signals detected by phosphor imager or X-ray film were then evaluated for the selection of potential substrates. We verified the potential substrates by on blot-phosphorylation *in vitro*. As a whole, our approach allows to shortlist candidate substrates of plant protein kinases for further analysis. Follow-up *in vivo* experiments are essential to evaluate their physiological relevance ***(13,25,26)***.

2. Materials

2.1. Purification of Recombinant Kinases under Native Conditions

1. Medium for culturing cells: 2YT or LB containing 100 µg/ml ampicillin, 15 µg/ml kanamycin and 2% glucose.
2. Isopropyl-β-D-thiogalactopyranoside, IPTG.
3. Native lysis buffer: 300 m*M* NaCl, 50 m*M* NaH_2PO_4, 10 m*M* imidazole, pH 8.0.
4. Native wash buffer: 300 m*M* NaCl, 50 m*M* NaH_2PO_4, 20 m*M* imidazole, pH 8.0.
5. Native elution buffer: 300 m*M* NaCl, 50 m*M* NaH_2PO_4, 250 m*M* imidazole, pH 8.0.
6. Lysozyme.
7. PMSF.
8. Ultrasonic homogeniser (Branson Ultrasonic, Danbury, CT).
9. 1 ml polypropylen columns (Qiagen, Hilden, Germany).
10. Bradford reagent (Bio-Rad Labratories GmbH, Munich).

2.2. Kinase Assay on Protein Microarrays

1. TBS+Tween (TBST): 10 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 0.1% (v/v) Tween.
2. Blocking solution: 2% bovine serum albumin, BSA (Sigma, St. Louis, MO) in TBST.
3. [γ^{33}-P]ATP, 250 µCi/ml (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).
4. CK2α buffer: 25 m*M* Tris–HCl pH 8.5, 10 m*M* $MgCl_2$, 1 m*M* DTT.
5. FAST™-slides (Whatman Schleicher & Schuell, Dassel, Germany).
6. Cover slide (Carl Roth GmbH, Karlsruhe, Germany).
7. X-ray cassette (Hypercassette, Amersham Pharmacia, Freiburg, Germany).
8. X-ray film (Kodak, Stuttgart, Germany).
9. Imaging screen (BAS-SR 2025, Fujifilm, Japan).
10. Phosphor imager (BAS-Reader-5000, Fujifilm, Japan).

3. Methods

3.1. Purification of Recombinant Kinases under Native Conditions

For the phosphorylation screening the kinase has to be soluble, active and pure, which means that no other kinase should be present. To achieve this a possibility would be a large scale production and purification of recombinant His-tagged protein kinases from cDNA expression libraries, e.g. a barley expression library constructed in the *E. coli* vector pQE30NST (accession number: AF074376; ***22***) or an Arabidopsis expression library in vector pQE30NASTattB (accession number: AY386205; ***23***) (*see also* ***24***). In order to express and purify protein kinases from these libraries we apply the following protocol:

For expression:

1. 50 ml falcon tubes were filled with 10 ml of medium for culturing cells.
2. Cultures were inoculated with bacteria from 384-well plates, which were stored at −80°C.
3. After overnight growth at 37°C with vigorous shaking the cultures were transferred into 300 ml Erlenmeyer flasks and 90 ml of prewarmed 2YT medium supplemented with 100 µg/ml of ampicillin, and 15 µg/ml of kanamycin were added. The incubation was continued until an OD of 0.7 was reached.
4. To induce protein expression, IPTG was added to a final concentration of 1 m*M*, and incubation was continued for 4 h at 37°C.
5. Cells were transferred into 50 ml falcon tubes (two for each culture), harvested by centrifugation at 1,900 × g for 10 min and stored immediately at −80°C.

For native protein purification (all of the following steps were performed at 0–4°C):

1. The frozen pellets were thawed on ice.
2. Cells were lysed in 0.5 ml of native lysis buffer supplemented with 0.25 mg/ml of lysozyme and 0.1 m*M* of PMSF.
3. Lysates were pooled and DNA was sheared with an ultrasonic homogeniser for 3 × 1 min at 50% power on ice.
4. Lysates were transferred into 1.5 ml Eppendorf-tubes and centrifugated at 20,000 × g, 4°C.
5. Supernatants were transferred into fresh Eppendorf-tubes.
6. Then 250 µl of NiNTA–agarose were added to bind the His-tagged proteins by shaking for 1 h at 300 rpm at 4°C.
7. The mixtures were transferred to 1 ml polypropylen columns.
8. The columns were washed with 10 bed volumes native wash buffer.
9. Proteins were eluted with 4 elution steps using 0.5 ml of native elution buffer each.
10. Proteins were mixed with glycerol (20% (v/v) end concentration) and stored at −80°C (*see* **Note 1**).

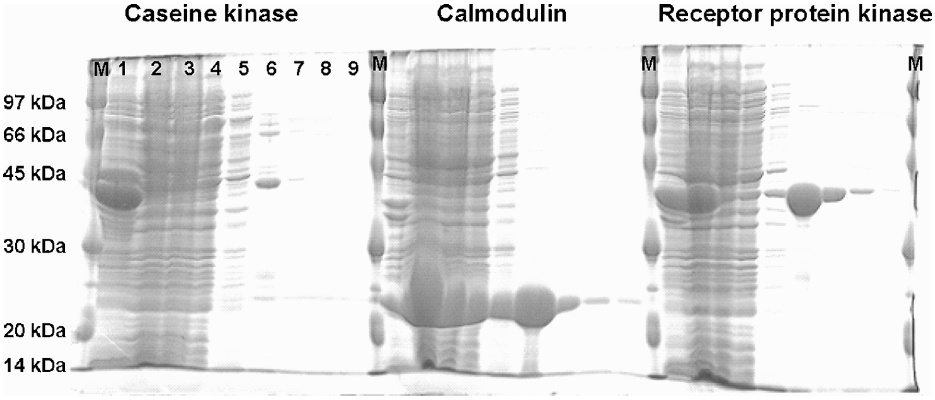

Fig. 1. 15% SDS-Page of two native purified kinases and calmodulin (Coomassie-stained). Nine samples of each purification step were separated: 1: pellet (insoluble fraction after centrifugation); 2: supernatant (soluble fraction after centrifugation); 3: first column flow-through; 4+5: first and second washing fraction; 6–9: eluates. M, molecular weight marker. Approximate size of the protein markers are shown at the left in kDa.

We would recommend collecting an aliquot from each centrifugation-, lysis-, wash- and elution step in order to control the efficiency of the purification steps using a polyacrylamide gel, e.g. 15% (*see* **Fig. 1**). Protein concentration was determined by Bradford assay *(27)*.

3.2. Kinase Assay on Protein Microarrays

We recommend checking the activity of the kinase prior to microarray experiments (*see* **Note 2**) using a known substrate as a positive control (*see* **Note 3**). Furthermore, prior to microarray experiments, one has to exclude that the kinase will phosphorylate 3′- or 5′-tags of the recombinant proteins, such as His-tags (*see* **Note 4**).

We intended to perform a qualitative signal evaluation after kinase assay. Therefore we have spotted proteins and controls (*see* **Note 3**) in quadruplicates on FAST™-slides (*see* **Note 5**) using a 10 × 10 spotting pattern (*see* **Note 6**) as demonstrated in **Fig. 2** *(22)*. Spotting pattern for further quantitative evaluation has been described recently *(23)*.

For kinase assay:

1. Microarrays, which were spotted the day before and stored at 4°C in a closed slide holder, were washed for 1 h in TBST with vigorous shaking at room temperature (*see* **Note 7**).
2. Washed microarrays were blocked for 1 h at room temperature with 2% BSA/TBST.
3. Blocked microarrays were placed on Whatman paper soaked with TBST to avoid drying of the micorarrays during the subsequent kinase reaction.

4. Microarrays were incubated with 250 µl kinase solution containing 13 µg/ml of CK2α and 25 µCi/ml of radioactive labelled [γ^{33}-P]ATP in CK2α buffer for 1 h at room temperature (*see* **Note 1**). This incubation was performed underneath a cover slide.
5. Six wash steps of 30 min each were performed in TBST.
6. Finally, the microarrays were dried using a microtiter plate centrifuge (e.g., Eppendorf AG, Hamburg, Germany: 5810R) or by manual fanning and transferred into an X-ray cassette.
7. Signal detection was performed by means of an X-ray film. The film was laid on the microarrays and the cassette was stored for a suitable time at −80°C (*see* **Note 8**). Afterwards, the film was developed in a dark chamber. Alternatively, the microarrays were exposed to an imaging screen, which was screened afterwards by a phosphor imager for signal detection (*see* **Note 8**).

Fig. 2 (left) shows a typical result of a phosphorylation screening experiment with CK2α after exposure of the microarrays to X-ray film. Quadruples of several proteins gave distinct signals on the film.

In order to determine the immobilisation of the recombinant His-tagged proteins on the microarrays we screened them with an anti-RGS-His_6 antibody as described in detail previously *(24)*. Nearly all of the spotted proteins were detectable (~95%) as shown in **Fig. 2** (right).

3.3. Selection of Potential Kinase Targets

For qualitative signal evaluation, the results of two independent experiments with different preparations of the kinase have been used.

The criteria for the selection of the potential kinase targets were:

1. A protein was considered as positive in one experiment if all 4 spots of the corresponding quadruplicate gave a distinct signal on the X-ray film or in the phosphor imager (*see* **Fig. 2**).
2. A protein was regarded as potential target protein if it attained positive results in both experiments.

Thus, for example 21 potential CK2α target proteins were identified out of nearly 800 different barley proteins *(22)*. The selection of potential targets using a threshold-based quantification system after phosphorylation on microarrays has been described in high throughput studies in Arabidopsis thaliana *(23)* as well as in yeast *(28)*.

3.4. Verification of Potential Phosphorylation Targets

As this microarray-based phosphorylation method is a screening tool to identify potential phosphorylation targets in vitro, we would recommend a verification of the identified targets by other methods. For such verifications various in vitro or in vivo approaches may be applied. A few of them have already been mentioned in the introduction.

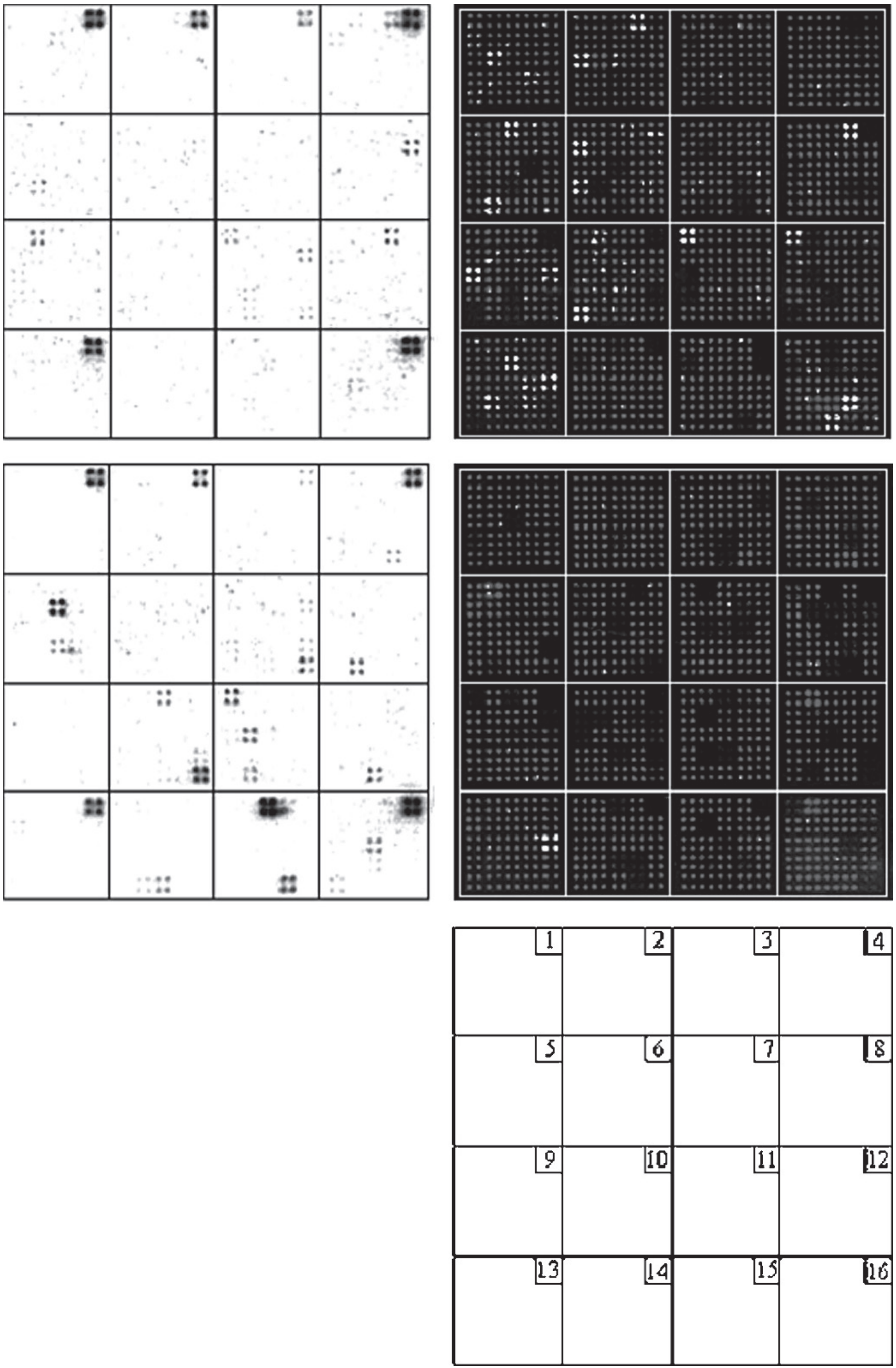
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Fig. 2.

We performed an on blot-phosphorylation assay to verify potential substrates:

1. Proteins were separated using an SDS–PAGE, e.g., 15%, followed by blotting the proteins on PVDF membranes. After blotting, gels were Coomassie stained.
2. Phosphorylation reaction was carried out on the blot membrane using reaction conditions as described for the microarray-based assay in an appropriate volume.

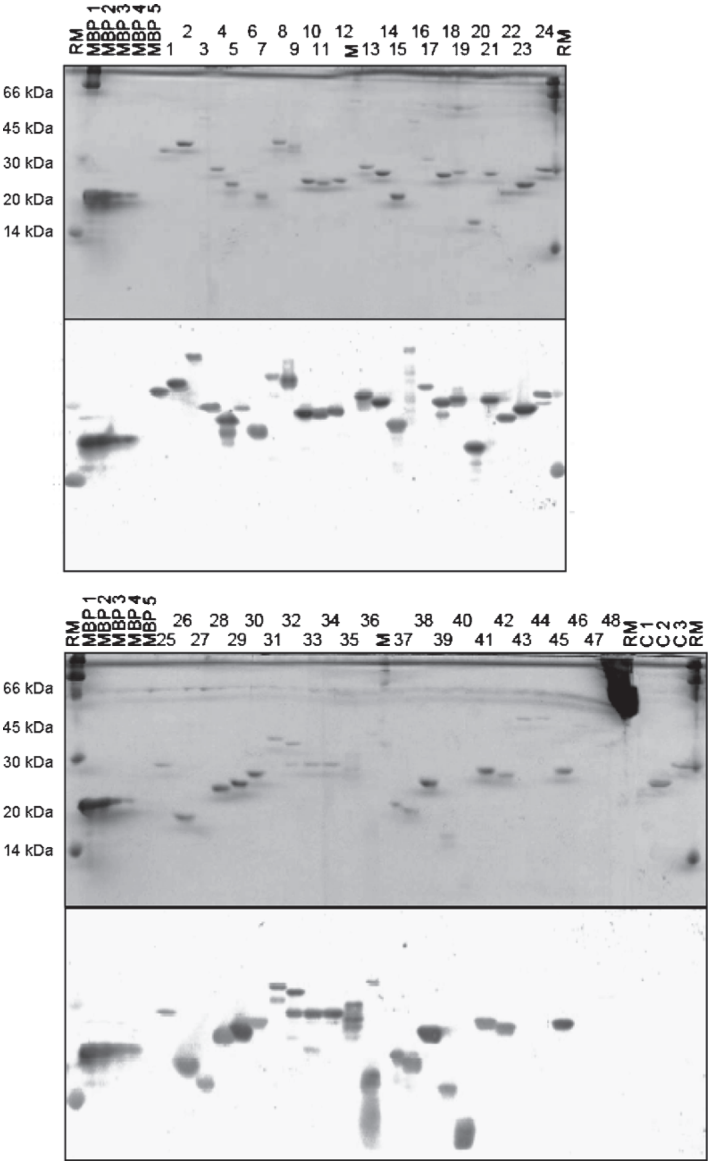
Fig. 3 shows a typical example of such an on blot-phosphorylation experiment. Fourty-eight proteins, which were identified in both microarray experiments as potential substrates using a MAP kinase were verified with this method. Nearly all of them were confirmed as shown in **Fig. 3**. We used the same positive controls as in the microarray experiments: different concentrations of myelin basic protein (MBP; artificial substrate of MAP kinases). For negative controls proteins were taken, which were tested in the microarray-based kinase assay and were not identified as potential substrates (*see* **Fig. 3**).

Other *in vitro* verification methods are phosphorylation assays performed in solution with subsequent detection of the phosphorylated proteins by SDS electrophoresis and autoradiography ***(8,23)***. Such assay has been used by Fukunaga and Hunter to verify candidate kinase substrates identified by phosphorylation screening of an expression phage cDNA library ***(8)***.

4. Notes

1. We recommend to store kinases at −80°C by using glycerol (end concentration 20%) or to lyophilise the kinases in their according buffer and to store them afterwards at −80°C. The lyophilised kinases are only being dissolved in ddH$_2$O shortly before use.
2. Prior to performing micorarray-based kinase experiments we recommend to test the activity of the prepared kinase by in gel assays ***(29)*** or kinase assays in solution ***(8)*** using a known substrate of the respective kinase (*see*

Fig. 2. Kinase- and immunoassay with 768 different barley proteins, immobilized on FAST™-slides (384 proteins in each field). Left: X-ray film image with the results of a representative CK2α-assay. Right: equal microarrays screened with an anti-RGS-His$_6$ antibody. The map below shows the positions of the 16 controls. The purified proteins and the controls were spotted in quadruplicates. Controls: 1, 4, 13, 16: HMG(1) (GenBank Acc.: CK124404), 0.29 mg/ml; 2: HMG(2) (GenBank Acc.: CK122788), 0.76 mg/ml; 3: HMG(3) (GenBank Acc.: CK124847), 0.28 mg/ml; 5: CK2α (GenBank Acc.: CK123377), 0.22 mg/ml; 6: other library kinase (GenBank Acc.: CK125548), 0.21 mg/ml; 7: human gapdh (His-tagged recombinant human glyceraldehyde-3-phosphate dehydrogenase), 0.36 mg/ml; 8: BSA, 20 pmol/µl in PBS; 9: native elution buffer; 10: denaturing elution buffer; 11: mouse anti-RGS-His$_6$ antibody, diluted 1:10 in PBS; 12: rabbit-anti-mouse IgG-Cy3 conjugate, diluted 1:25 in PBS; 14: H$_2$O; 15: PBS. (Reprinted with permission from ***ref. 22***. Copyright 2004 Elsevier Science).


RM
MBP 1
MBP 2
MBP 3
MBP 4
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1 2 3 4 5 6 7 8 9 10 11 12 M 13 14 15 16 17 18 19 20 21 22 23 24
RM
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MBP 3
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14 kDa

Fig. 3. Verification of kinase substrates. 15% SDS-PAGE gels of potential kinase substrates (upper panel) and autoradiograms of these proteins after blotting and kinase reaction (lower panel). Gels were Coomassie-stained after electrophoresis and blotting. MBP1 – MBP5: positive controls, MBP in various dilution steps (MBP1: 2,000 ng/µl; MBP2: 1,000 ng/µl; MBP3: 500 ng/µl; MBP4: 250 ng/µl; MBP5: 125 ng/µl). 1 – 48: potential kinase substrates identified in the microarray experiments. C1-C3: negative controls; these are proteins, which were not identified as potential substrates using microarrays. RM: rainbow marker; M: molecular weight marker. Approximate size of the protein markers are shown at the left in kDa.

Note 3). The optimal conditions for kinase activation (e.g., optimal buffer conditions, kinase concentration and incubation time of the kinase) must be determined experimentally for each individual kinase, if they are not known from the literature.

3. The use of a positive control is recommendable (e.g., a known substrate of the kinase). In studies with barley CK2α ***(22)*** we used as positive controls different barley proteins, which share a strong homology with different plant HMG (high mobility group) proteins. These proteins are well known targets of the CK2α ***(30,31)***. In another study we analyzed different Arabidopsis MAP kinases ***(23)***. In this case a known artificial substrate, the myelin basic protein (MBP), was used as a positive control.
4. Recombinant proteins often contain 3'- or 5'- tags which are encoded by the cloning vector besides the coding sequence of the respective protein. Phosphorylation of these tags may result in false-positive results and should therefore be excluded prior to microarray experiments. One possibility is to perform test assays in solution with the active kinase using some recombinant proteins which are known to be no substrates of the respective kinase and which were expressed the same manner (same tags) as the recombinant proteins used for the microarray experiments. These selected proteins should yield negative results in the test.
5. With respect to surface coatings we tried to phosphorylate immobilized denatured proteins on FAST™-slides (coated with a nitrocellulose-derived polymer) and epoxy slides. We detected phosphorylation only on FAST™-slides although His-tagged proteins were detectable with anti-RGS-His_6 antibody on both surfaces. In other studies, nanowells ***(19)***, BSA-NHS (BSA-N-hydroxysuccinimide) slides ***(18)***, or SAM2 (streptavidin-coated membrane) slides ***(32)*** have been used as surfaces in microarray-based kinase assays. We reviewed different micorarray surfaces for different microarray applications including phosphorylation studies ***(33)***.
6. The maximum spot density, at these individual spots can still be differentiated in a phosphorylation assay, is highly dependent on the resolution of the phosphor imager, which scanned the imaging plates. For the determination of this density we recommend the use of commercially available protein kinase (e.g., PKA from New England Biolabs GmbH). Our experience is, when using a scanner with a resolution of 10 µm (e.g., BioImager FLA 8,000, Fujifilm, Japan) in combination with spotting patterns up to 11 × 11 (spot distance: 410 µm), that we received sufficient resolution to evaluate the radioactive signals. Applications of higher spotting pattern (14 × 14-pattern with a spot distance of 321 µm; 15 × 15-pattern with a spot distance of 300 µm) were not suitable for radioactive detection, as signals of intense spots were interfering with neighbouring spot signals. This lead

to faulty analysis of the signal intensity of neighbouring proteins as well as faulty background corrections.

7. This washing step in TBST was performed to remove urea from the microarrays, as we recognised that the urea reduces the activity of the kinase in the microarray assay.
8. For the detection of radioactive signals with X-ray film, the film was laid onto the microarrays, which have been covered with cling film. The exposition time is dependent on the signal intensity and can vary between 30 min to a few days. The sensitivity of the X-ray films can cause problems, as the blackening of the films is only in a very limited area linear and proportional to the signal intensity. For the detection with a phosphor imager the imaging plates are laid onto the covered microarrays and are then scanned. Detection by phosphor imager is 10–100 × more sensitive than by X-ray films and therefore it is possible to get faster results and/or detect poor signals respectively. Furthermore, the system has a higher dynamic range compared to the X-ray film. Strong signals as well as poor signals are detected within one exposition and the linear signal-to-intensity range has a dimension over 5 (100,000:1). This shows that the phosphor imager is of better use for quantification of radioactive signals than X-ray films.

Acknowledgments

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Two-Dimensional Phosphopeptide Mapping

Hikaru Nagahara, Robert R. Latek, Sergei A. Ezhevsky,
and Steven F. Dowdy

1. Introduction

A major mechanism that cells use to regulate protein function is by phosphorylation and/or dephosphorylation of serine, theonine and tyrosine residues. Phosphopeptide mapping of these phosphorylated residues allows investigation into the positive and negative regulatory roles these sites may play *in vivo*. In addition, phosphopeptide mapping can uncover the specific phosphorylated residue and hence, kinase recognition site, thus helping in the identification of the relevant kinase(s) and/or phosphatase(s).

Two-Dimensional (2D) Phosphopeptide mapping can utilize in vivo and in vitro ^{32}P labeled proteins ***(1–6)***. Briefly, ^{32}P labeled proteins are purified by SDS-PAGE, transferred to a nitrocellulose filter and digested by proteases or chemicals. The phosphopeptides are then separated by electrophoresis on thin layer cellulose (TLC) plate in the first dimension followed by thin layer chomatography in an organic buffer in the sond dimension. The TLC plate is then exposed to autoradiographic (ARG) film or phosphor-imager screen and the position of the ^{32}P containing peptides are thus identified. Specific phosphopeptides can then be excised from the TLC plate and analyzed further by amino acid hydrolysis to identify the specific phosphorylated residue(s) and/or by manual amino-terminal sequencing to obtain the position of the phosphorylated residue(s) relative to the protease cleavage site ***(3)***. In addition, mixing *in vivo* with *in vitro* ^{32}P-labeled proteins can yield confirmation of the specific phosphorylated residue and the relevant kinase.

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2. Materials

2.1. Equipment

1. Multiphor II horizontal electrophoresis apparatus (Pharmacia).
2. Power pack capable of 1000 V constant.
3. Refrigerated circulating water bath.
4. Thin layer chomatography (TLC) chamber, ~30 L × 10 W × 28H cm, and internal stand.
5. Speedy-vac or lyophilizier.
6. Shaking water bath.
7. SDS-PAGE apparatus.
8. Semi-Dry blotting apparatus (Owl).
9. Small fan.
10. Rotating wheel or apparatus.

2.2. Reagents

1. Phosphate-free tissue culture medium.
2. Phosphate-free dialyzed fetal bovine serum (FBS). Alternatively, dialyze 100 ml FBS against 4 L dialysis buffer for 12 h and repeat two more times using 10,000 MWCO dialysis tubing. Dialysis buffer: 32 g NaCl, 0.8 g KCl, 12 g Tris in 4 L, pH to 7.4 with HCl.
3. $^{32}PO_4^{2-}$ orthophosphate, 3–5 mCi per tissue culture dish.
4. Protein extraction buffer (ELB): 20 m*M* HEPES (pH 7.2), 250 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, 0.1–0.5% NP-40 or Triton X-100, 1 µg/ml Leupeptin, 50 µg/ml PMSF, 1 µg/ml Aprotitin, and containing the following phosphatase inhibitors: 0.5 m*M* NaP_2O_7, 0.1 m*M* $NaVO_4$, 5.0 m*M* NaF.
5. Rabbit anti-Mouse IgG.
6. Killed *S. aureus* cells (Zyzorbin).
7. Protein A agarose.
8. 2× Sample Buffer: 100 m*M* Tris-HCl (6.8), 200 m*M* DTT, 4% SDS,0.2% bromophenol blue, 20% glycerol.
9. Protein Transfer buffer: 20% methanol, 0.037% SDS, 50 m*M* Tris, 40 m*M* glycine.
10. 50 mM NH_4HCO_3, made fresh each usage from powder.
11. 0.5% (w/v) Polyvinyl pyrolidone-360 in 100 m*M* acetic acid.
12. Sequencing grade trypsin (Boehinger Mannheim), resuspend 100 µg in 1.5 ml fresh 50 m*M* NH_4HCO_3, store 10 µg/150 µl aliquots at −20°C.
13. Performic acid: Mix 1 vol hydrogen peroxide with 9 vol formic acid. Incubate for 1.5 h on ice.
14. Scintillation fluid.
15. Thin-layer chomatography cellulose plastic-backed plates, 20 × 20 cm (Bakerflex/VWR).
16. pH 1.9 Electrophorese Running Buffer: 50 ml 88% formic acid, 56 ml acetic acid and 1894 ml H_2O. Do not adjust pH.

17. Electrophoresis color marker: 5 mg/ml DNP-lysine, 1 mg/ml xylene cyanol FF in 50 µl n-butanol, 25 µl pyridine, 25 µl acetic acid, 1.9 ml H_2O.
18. TLC chamber buffer: 75 ml n-butanol, 50 ml pyridine, 15 ml glacial acetic acid, 60 ml H_2O.

3. Methods

3.1. ^{32}P Orthophosphate Labeling

1. For in vivo ^{32}P orthophosphate labeling of cellular proteins, preplate approximately 1×10^6 cells in a 10 cm dish. Rinse adherent cells thee times with 5 ml phosphate-free media. Suspension cells can be rinsed and collected by centrifugation at ~1800 rpm for 5 at RT or 30°C, aspirate the media and repeat as above. Add 3–5 mCi of ^{32}P ortho phosphate in 3.5 ml of phosphate-free media containing 10% dialyzed serum to the 10 cm dish and incubate cells at 37°C for 4–6 h (*see* **Note 1**).
2. Aspirate the ^{32}P containing media with a plastic pipette, transfer supernatant waste into a 50 ml conical disposable tube. Rinse the cells twice with 10 ml PBS(-) and combine with ^{32}P media waste in 50 ml tube and dispose of properly.
3. Add 1 ml ice cold extraction buffer (ELB; 7) and place dish on a flat bed of ice, behind a shield. Tilt dish slightly every 30 s for 3'–5' to continually cover the cells. Collect cellular lysate and debris by tilting dish approximately 30° using a P-1000 pipetman tip and transfer to 1.5 ml eppendorf tube and mix. Alternatively, adherent cells can be released by trypsin/EDTA addition, collected and washed twice in media containing serum to inactivate the trypsin. After the final centrifugation, add 1 ml ELB, mix by using a P-1000 tip and transfer to an eppendorf tube. Place tube on ice for 20'–25' with occasional mild inverting (*see* **Note 2**).
5. Spin out insoluble particular matter from the cellular lysate in microfuge at 12 K, 4°C, for 10'. Transfer supernatant to new eppendorf tube and preclear lysate by the addition of 50 µl killed *S. aureus* cells, cap tube and place on rotating wheel at 4°C for 30'–60'.
6. To remove *S. aureus* cells, centrifuge lysate at 12 K, 4°C for 10 . Transfer lysate to fresh eppendorf tube, making sure to leave the last 50 µl or so of lysate behind with the pellet. The presence of contaminating *S. aureus* cells in this portion of the sample will reduce the amount of immunocomplexes recovered if included.

3.2. Immunoprecipitation and Transfer of ^{32}P-Labeled Protein

1. Add primary antibody to the precleared lysate supernatant, usually 100–200 µl in the case of hybridoma supernatants and 3–5 µl of commercially purified antibodies. Add 30 µl protein A agarose beads, cap the tube, and place on a wheel at 4°C for 2–4 h to overnight. If primary mouse antibody isotype is IgG1 or unknown, add 1 µl Rabbit anti-Mouse IgG to allow indirect binding of the primary antibodies to the protein A agarose *(7)*.
2. After incubation with primary antibody, perform a "1 g spin" by placing the tube on ice for 15', aspirate the supernatant to just above the protein A agarose bed level.

Stop aspirating at the top of the agarose beads, avoiding drying the beads. This supernatant waste is still highly radioactive. Wash the agarose beads by addition of 1 ml ice cold ELB, cap and invert tube several times and centrifuge at 12 K, 4°, 30 s. Aspirate supernatant and repeat two to thee more times. Again, avoid drying the beads.

3. After the final 30 s spin, aspirate supernatant off of the protein A beads until just dry, add 30 µl of 2× Sample buffer. Boil sample for 5 min, centrifuge 12 K for 10 s, cool tube on ice and load immunocomplexes onto a SDS-PAGE *(8)*.
4. After running the SDS-PAGE, separate the glass plates and trim down the gel with a razor blade by removing the stacking gel and any excess on the sides or bottom. Measure the trimmed gel size and cut six sheets of 3 MM Whatman filter paper and one sheet of nitrocellulose (NC) filter to the same size.
5. Soak two sheets of cut 3 MM filter paper in transfer buffer and place on semi-dry transfer unit. This can be done by filling a tupperware-like container with transfer buffer and dipping the 3 MM paper into it. Place one soaked 3 MM sheet on the back of gel and rub slightly to adhere to gel. Then invert the glass plate with the gel-3 MM still stuck to it and peel gel-3 MM away from the glass with the use of a razor blade. Place it 3 MM side down onto the soaked 3 MM sheets on the semi-dry unit. Soak the cut NC filter with transfer buffer and place it on top of gel followed by thee presoaked 3 MM sheets on top of filter. Gently squeegee out bubbles and excess buffer with the back of your little finger or by rolling a small pipette over the stack. It is important to squeegee out the bubbles, but avoid excess squeegeeing that would result in drying the stack. Mop up the excess buffer on the sides of the stack present on the transfer unit. Place the top on the transfer unit. In this configuration, the bottom plate is the cathode (negative) and the top the anode (positive).
6. Transfer the ^{32}P-labeled proteins to the NC filter at 10 Volts constant for 1.5 to 2 h. The starting current will vary from ~180 to 300 mAmp depending on the surface area of the gel and will drop to around 80 to 140 mAmp by the end of the transfer.

3.3. Trypsinization of Protein on Nitrocellulose Filter

1. Following transfer of ^{32}P-labeled proteins to NC filter, open the transfer unit and using a pair of filter forceps place the NC filter, protein side down onto saran-wrap and cover. Expose the saran-wrap covered NC filter to ARG-film for approximately 1 h to 2 h with protein side towards the film. The length of the exposure will vary with the abundance of ^{32}P content and protein, and with the efficiencies of recovery from immunoprecipitation and transfer. Radioactive or luminescent markers are needed on the filter to determine the orientation, position and alignment of the NC filter with respect to the ARG film. Using the markers, line up your filter on top of its ARG. This is best done on a light box. Use a razor blade to excise the slice of NC filter that corresponds to the ^{32}P-labeled protein band.
2. Place the NC filter slice into an eppendorf tube, add ~200 µl of 0.5% PVP-360 in 100 mM acetic acid, cap tube and incubate in a shaking water bath at 37°C for 30 min (*see* **Note 3**).

3. Wash the filter slice five times with 1 ml H_2O and then twice with 1 ml fresh 50 m*M* NH_4HCO_3.
4. Add 10 µg of trypsin in 150 µl of 50 mM NH_4HCO_3 to the NC filter slice. Incubate in a 37°C shaking water bath over-night. Following this incubation, spike the digestion of the NC filter with another 10 µg of trypsin in 150 µl of 50 m*M* NH_4HCO_3 and incubate at 37°C for an additional 4 h.
5. Vortex the tube containing the NC filter/trypsin sample for 1 min, centrifuge at 12 K for 30 s. Transfer the supernatant to a new eppendorf tube. Wash the NC filter slice by addition of 300 µl H_2O, vortex 1 min, centrifuge at 12 K for 30 s, and combine the supernatants.
6. Freeze the trypsinized ^{32}P-labeled peptide sample on dry ice and then completely dry in a speedy-vac (without heat). This generally takes about 4 h to complete. Prepare the performic acid for the oxidation step **(step 8)**.
7. After the sample has been completely dried, add 50 µl of ice cold performic acid and place on ice for 1 h. Stop the reaction by addition of 400 µl H_2O to the sample, followed by freezing on dry ice and then drying in a speedy-vac.
8. Resuspend the sample in 8–10 µl of H_2O. Determine the level of radioactivity by counting 0.5 µl of the sample on a scintillation counter. Usually a total of ≥2000 cpm is sufficient for 2D phosphopeptide mapping.

3.4. Phosphopeptide Separation: First and Second Dimensions

1. Mark the origin on the thin-layer cellulose (TLC) plate by lightly touching a pencil to the TLC plate at the position indicated in **Fig. 1**. Apply multiple 0.5 µl aliquots of the trypsinized ^{32}P-labeled peptides to the origin to achieve ≥2000 cpm. Dry the TLC plate thoroughly between each aliquot application by use of a gentle fan. Pay particular attention to adding each subsequent aliquot to the same small area at the origin. Add 0.5 µl of the color marker 3 cm from the top edge of the TLC plate **(Fig. 1)** and then dry the TLC plate under a fan for an additional 30–60 min.
2. Prepare the Multiphor II apparatus for electrophoresis. Place the Multiphor II in a cold room, connect the cooling plate to the cooling circulator bath hoses and precool to 5°C. Prepare and chill the pH 1.9 running buffer and add to Multiphor II buffer tanks. Insert the electrode paddles into innermost chambers, and attach the wire connections. We have used the IEF electrodes in direct contact with the cellulose plate; however, using the paddles provided and wicking buffer onto the plate yields the best results (*see* **Figs. 1** and **2**). Place the cooling plate into the Multiphor apparatus. Add 1 L of prechilled pH 1.9 running buffer to each chamber of the Multiphor II. (These instructions are provided with the Multiphor II unit.)
3. Place the loaded TLC plate on top of the cooling plate. To dampen the TLC plate with buffer, first cut a 21 × 21 cm piece of 3 MM Whattman paper and make an approximately 1 cm hole at the origin by puncturing the 3 MM paper with a pencil. Soak the cut 3 MM paper in pH 1.9 running buffer, blotting it between two sheets of dry 3 MM paper, and then placing it over the loaded TLC plate sitting on top of the cooling plate. Slowly pipette running buffer onto the 3 MM paper until the entire cellulose plate is damp beneath, avoid excessive puddling. Remove the paper

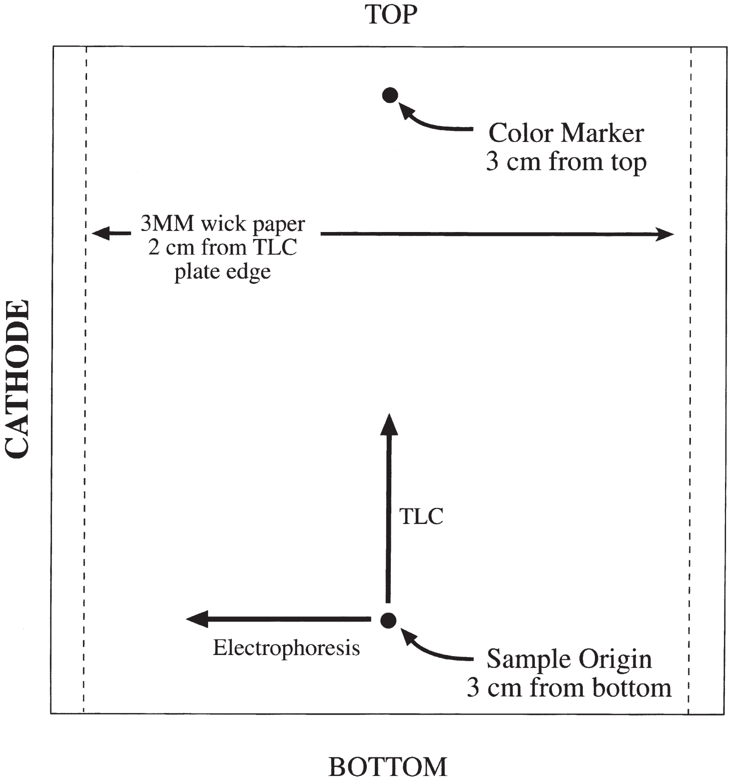
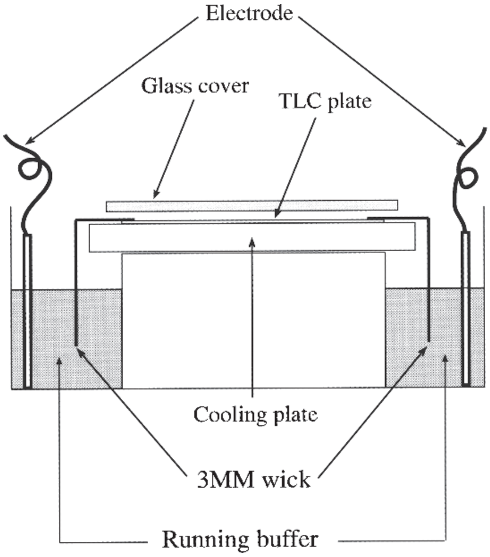

TOP
Color Marker
3 cm from top
3MM wick paper
2 cm from TLC
plate edge
CATHODE
ANODE
TLC
Electrophoresis
Sample Origin
3 cm from bottom
BOTTOM

Fig. 1. The location of the origin, anode, cathode and color dye marker dye relative to each other on the 20 × 20 cm TLC plate are depicted (*see* **Subheading 3.4, steps 1** and **2**.

and wick a single piece of 13 × 21 cm buffer-soaked 3 MM filter paper from the buffer chamber onto the 2 cm outer edge on both sides of the plate (*see* **Fig. 2**). Be sure to fold the paper neatly over the edge of the cooling plate and to make sure that it is evenly contacting the TLC plate. Place the glass cover over the TLC plate touching/resting on the 2 cm overhang of the 3 MM paper wicks. Attach the Multiphor II cover.

4. Electrophorese the peptides on the loaded TLC plate at 1000 V constant for 28–30 min. The run time may be increased up to 38 min. If further separation of ^{32}P-labeled peptides in this dimension is required, the run time may be increased up to 38 min.
5. Following the first dimension separation by electrophoresis, remove the TLC plate from the Multiphor II apparatus and dry for 1 h with a fan.
6. Place the TLC plate on a stand in a thin-layer chomatography chamber pre-equilibrated for 48 h in chomatography buffer. The TLC buffer should cover approximately 1 cm of the bottom of the TLC plate when placed on the stand.

Fig. 2. A cross stional view of the Multiphor II apparatus. Loaded TLC plate, 3 MM Whatman filter paper wicks, and glass cover plate are depicted in the running position (*see* **Subheading 3.4, steps 2** and **3**).



Leave the TLC plate in the chamber until the solvent line diffuses to the dye position at the top of the TLC plate, 3 cm below the top of the plate. This usually takes 7–8 h (*see* **Note 4**).

7. Remove the TLC plate from the chamber and dry for >1 h. Expose the TLC plate to ARG-film or a phosphor-imager screen overnight and develop. If the signal is too weak, expose for 5 to 7 days. The use of a phosphor-imager greatly diminishes the length of time required to obtain a 2D phosphopeptide map (*see* **Fig. 3**).

4. Notes

1. We routinely label 1 × 10^6; however, depending on the abundance of the specific protein of interest and on the number of phosphorylation sites this number may vary from 1 × 10^5 to 1 × 10^7 cells. In addition, adherent and non adherent cells may both be labeled in suspension. The cells can be labeled with ^{32}P orthophosphate in a 15 or 50 ml disposable conical tube or T-flask. Please note that the activity of kinase(s)/phosphatase(s) present in adherent cells may be altered when labeling in suspension. Dish size is not important as long as enough media is added to cover the bottom of the dish/flask. Attempt to achieve a final ^{32}P orthophosphate concentration of 1.0 to 1.3 mCi/ml of media.
2. We use ELB to lyse cells and generate cellular extracts; however, any extraction buffer containing Triton X-100, SDS, DOC, NP-40 or similar detergent that will lyse cells will suffice. If a strong background is observed following the SDS-PAGE, transferring the immobilized protein A agarose

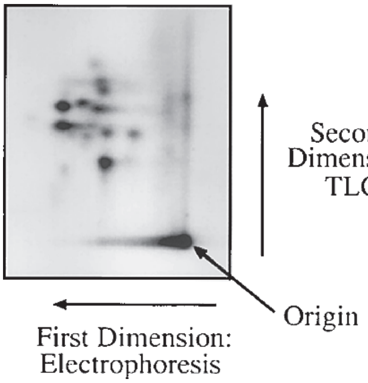


Fig. 3. 2-D phosphopeptide map of the retinoblastoma tumor suppressor gene product (pRb) labeled with ^{32}P phosphate *in vivo*. pRb contains thirteen cyclin-dependent kinase (cdk) phosphorylation sites, hence the complexity of the phosphopeptide map. Note the presence of several levels of ^{32}P intensity associated with specific peptides. This can arise by a number of mechanisms, including *in vivo* site preferences and/or accessibility of the nitrocellulose immobilized ^{32}P-labeled protein to trypsin. The origin, first and sond dimension runs are as indicated (*see* **Subheading 3.4, step 7**).

immunocomplexes to a new eppendorf tube prior to the final wash and centrifugation step can result in a reduced background with minimal loss of specific signal.

3. When running multiple lanes of the same ^{32}P-labeled protein immunocomplexes on SDS-PAGE treat each lane/NC filter slice separately. The trypsinized peptides from as many as five NC filter slices can be combined together, then frozen on dry ice and dried.
4. Seal the top of the TLC chamber with vacuum grease and minimize the amount of time the lid is off of the chamber. Pre-equilibrate chamber for >48 h prior to use. We routinely change the TLC chamber buffer every 8 weeks. Poor separation in the sond dimension is usually indicative of buffer alterations due to evaporation and/or hydration.
5. The procedures described in this chapter can be stopped at the following steps: (1) when the immunocomplexes are in 2× sample buffer following the immunoprecipitation; (2) after lyophilization following trypsinization of the NC filter slices; and (3) after drying the TLC plate following the first dimension eletrophoresis.

Acknowledgments

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Identification of Proteins Modified by Protein (D-Aspartyl/L-Isoaspartyl) Carboxyl Methyltransferase

Darin J. Weber and Philip N. McFadden

1. Introduction

The several classes of *S*-adenosylmethionine-dependent protein methyltransferases are distinguishable by the type of amino acid they modify in a substrate protein. The protein carboxyl methyltransferases constitute the subclass of enzymes that incorporate a methyl group into a methyl ester linkage with the carboxyl groups of proteins. Of these, protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase, EC 2.1.1.77 (PCM) specifically methyl esterifies aspartyl residues that through age-dependent alterations are in either the D-aspartyl or the L-isoaspartyl configuration ***(1,2)***. There are two major reasons for wishing to know the identity of protein substrates for PCM. First, the proteins that are methylated by PCM in the living cell, most of which have not yet been identified, are facets in the age-dependent metabolism of cells. Second, the fact that PCM can methylate many proteins in vitro, including products of overexpression systems, can be taken as evidence of spontaneous damage that has occurred in these proteins since the time of their translation.

The biggest hurdle in identification of substrates for PCM arises from the extreme base-lability of the incorporated methyl esters, which typically hydrolyze in a few hours or less at neutral pH. Thus, many standard biochemical techniques for separating and characterizing proteins are not usefully applied to the identification of these methylated proteins. In particular, the electrophoresis of proteins by the most commonly employed techniques of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results in a complete loss of methyl esters incorporated by PCM, owing to the alkaline pH of the buffers employed. Consequently, a series of systems employing polyacrylamide gel

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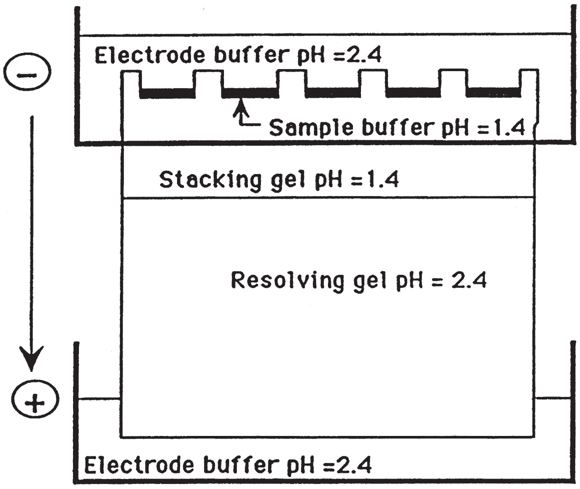

Fig. 1. Schematic of acidic discontinuous gel system. The system employs a pH 1.4 stacking gel on top of a pH 2.4 resolving gel with chloride as the leading ion and acetate as the trailing ion to stack the proteins tightly. The presence of the anionic detergent SDS allows the separation of proteins on the basis of molecular weight. The low pH preserves labile protein methyl esters, and so allows the identification of age-altered substrates of PCM.

electrophoresis at acidic pH have been utilized in efforts to identify the substrates of PCM. A pH 2.4 SDS system *(3)* using a continuous sodium phosphate buffering system has received the most attention ***(1,3–14)***. The main drawback of this system is that it produces broad electrophoretic bands. Acidic discontinuous gel systems using cationic detergents, ***(15)***, have proven useful in certain situations ***(16–21)*** and can be recommended if the cationic detergent is compatible with other procedures that might be utilized by the investigator (e.g., immunoblotting, protein sequencing). Recently, we have developed an electrophoresis system that employs SDS and an acidic discontinuous buffering system (**Fig. 1**). This procedure results in sharp electrophoretic bands and would be a good choice for investigators wishing to adhere to SDS as the anionic detergent. This system is described below, and examples of its ability to resolve proteins are provided.

2. Materials

2.1. Equipment

1. Slab gel electrophoresis unit: We have used the mini-gel electrophoresis units from Idea Scientific (Minneapolis, MN) (10 × 10 × 0.1 cm) with great success, as well as the Sturdier large-format gel system from Hoeffer Scientific Instruments (San Francisco, CA) (16 × 18 × 0.15 cm).
2. Electrophoresis power supply, constant current.

3. X-ray film and photo darkroom.
4. Scintillation counter.

2.2. Reagents

1. 40% (w/v) Acrylamide stock solution containing 37:1 ratio of acrylamide to *N,N′*-methylene-bis-acrylamide (Bio-Rad, Richmond, CA).
2. Resolving gel buffer: 0.1 *M* NaH_2PO_4 (Sigma, St. Louis, MO), 2.0 % SDS (United States Biochemical, Cleveland, OH [USB], ultrapure), 6 *M* urea (ultrapure) pH 2.4, with HCl.
3. Modified Clark and Lubs buffer (C & L buffer): Add 25.0 mL of 0.2 *M* NaCl to 26 mL of 0.2 *M* HCl, and bring to a final volume of 0.1 L. The buffer pH should be ~1.4 ***(22)*** (*see* **Note 1**).
4. Stacking gel buffer (2X): 2.0% (w/v) SDS, 6.0 *M* urea and C & L buffer such that the C & L buffer makes up 66% (v/v) of the total volume with the remaining 34% consisting of water and other buffer components. Buffer will be 0.033 *M* in NaCl. Readjust pH to 1.4 with HCl.
5. Sample solubilization buffer (2X): 2.0% (w/v) SDS, 6.0 *M* urea, 10% glycerol ([USB], ultrapure), 0.01% pyronin Y dye (Sigma), and C & L 33% (v/v) of the total volume, with the remaining 67% consisting of water and other buffer components. Buffer will be 0.0165 *M* in NaCl. Readjust pH to 1.4 with HCl.
6. Electrode buffer (1X): 0.03 *M* NaH_2PO_4, 0.1% SDS, 0.2 *M* acetate, pH 2.4 with HCl.
7. Gel polymerization catalysts: 0.06% $FeSO_4$, 1.0% H_2O_2, 1.0% ascorbic acid, prepared fresh in separate containers.
8. Colloidal Coomassie G-250 protein stain stock solution: 125 g ammonium sulfate, 25 mL 86% phosphoric acid, 1.25 g Coomassie brilliant blue G-250 (Sigma), deionized water to 1.0 L. The dye will precipitate, so shake well immediately before use. Stable indefinitely at room temperature ***(11)***.
9. Destain solution: 10% (v/v) acetic acid.
10. Fluorography solution: 1.0 *M* sodium salicylate brought to pH 6.0 with acetic acid ***(23)***.
11. X-ray film: Kodak X-Omat AR or equivalent.

2.3. Gel Recipes

2.3.1. 12% Acrylamide Resolving Gel, pH 2.4

The following volumes are sufficient to prepare one 7.5 × 10.5 × 0.15 cm slab gel:

2X Resolving gel buffer	3.75 mL
40% Acrylamide (37:1)	2.25 mL
0.06% $FeSO_4$	0.06 mL
1.0% Ascorbic acid	0.06 mL
0.3% H_2O_2	0.06 mL

2.3.2. 4.0% Stacking Gel, pH 1.4

The following volumes are sufficient to prepare one 3.0 × 10.5 × 0.15 cm stacking gel:

2X Stacking gel buffer	3.75 mL
40% Acrylamide (37:1)	0.75 mL
0.06% $FeSO_4$	0.06 mL
1.0% Ascorbic acid	0.06 mL
0.3% H_2O_2	0.06 mL

3. Methods

3.1. Sample Solubilization

1. An equal volume of 2X solubilization buffer is added and the samples are heated in a 95°C heating block for no more than 30 s (*see* **Note 2**).
2. To separate samples under reducing conditions, it is critical to add any reducing agent before addition of the solubilization buffer. Up to 10 µg/gel protein band can be resolved with this system; total sample loaded in a single well should not exceed about 100 µg.

3.2. Gel Preparation

3.2.1. Resolving Gel

1. Add all the components listed under **Subheading 2.3.1**, except the 0.3% H_2O_2, together in a small Erlenmeyer side-arm flask.
2. Degas the solution for at least 5.0 min using an in-house vacuum.
3. Assemble together gel plates and spacers that have been scrupulously cleaned and dried.
4. Using a pen, make a mark 3.0 cm from the top of the gel plates to denote the space left for the stacking gel.
5. To the degassed gel solution, add the H_2O_2 catalyst. Gently mix solution by pipeting the solution in and out several times. Avoid introducing air bubbles into the solution.
6. Quickly pipet the acrylamide solution between the glass gel plates to the mark denoting 3.0 cm from the top of the gel plates.
7. Carefully overlay the acrylamide solution with about a 2.0-mm layer of water-saturated butanol using a Pasteur pipet, so that the interface will be flat on polymerization.
8. Allow the gel to polymerize at room temperature until a distinct gel–butanol interface is visible.
9. After polymerization is complete, pour off the overlay, and gently rinse the top of the gel with deionized water. Invert the gel on paper towels to blot away any remaining water between the gel plates.

3.2.2. Stacking Gel

1. Add all the components listed under **Subheading 2.3.2**, except the 0.3% H_2O_2, together in a small Erlenmeyer sidearm flask.

2. Degas the solution for at least 5.0 min using in-house vacuum.
3. Soak the well-forming combs in the H_2O_2 catalyst solution while preparing the stacking gel.
4. To the degassed gel solution, add the H_2O_2 catalyst. Gently mix solution by pipeting the solution in and out several times. Avoid introducing air bubbles into the solution.
5. Quickly pipet the acrylamide solution between the glass gel plates to the very top of the gel plates.
6. Remove the combs from the H_2O_2 catalyst solution, shake off some of the excess solution, and insert the comb in between the gel plates by angling the comb with one hand and guiding the comb between the plates with the other hand.
7. Ensure the comb is level relative to the top of the resolving gel and that no bubbles are trapped under the comb.
8. After the stacking gel has completely polymerized, carefully remove the comb. Remove any unpolymerized acrylamide from each well by rinsing with deionized water (*see* **Note 3**).

3.3. Electrophoresis

1. Assemble the gel in the electrophoresis unit.
2. Add sufficient electrode buffer to cover the electrodes in both the upper and lower reservoirs.
3. Remove any air bubbles trapped under the bottom edge of the plates with a bent 25-gage needle and syringe containing electrode buffer.
4. Rinse each well with electrode buffer immediately before adding samples.
5. Load all wells with 40–60 µL of protein samples; load 1X solubilization buffer into any empty wells.
6. Run the gels at 15 mA, constant current, at room temperature for 4–6 h, or until proteins of interest have been adequately resolved (*see* **Note 4**).
7. Fix the gel in 12% (v/v) TCA with gentle shaking for 30 min.
8. Pour off fixative, and mix 1 part methanol with 4 parts of colloidal Coomassie G-250 stock solution. Slowly shake gel with staining solution for ~12 h (*see* **Note 5**).
9. Pour off staining solution. Any nonspecific background staining of the gel can be removed by soaking the gel in 10% (v/v) acetic acid. Little destaining of protein bands occurs even after prolonged times in 10% acetic acid.

3.4. Detection of Radioactively Methylated Proteins

Several protocols exist for radioactively methylating proteins with PCM ***(2,16)***. Following electrophoresis, gel bands containing radioactively labeled proteins can be detected by fluorography or scintillation counting of gel slices.

3.4.1. Fluorography of Gels

1. If gels have been stained, they are destained using 10% methanol/7% acetic acid to decolorize the gel bands.
2. Expose the gel to fluorography solution for 30 min at room temperature with gentle shaking.

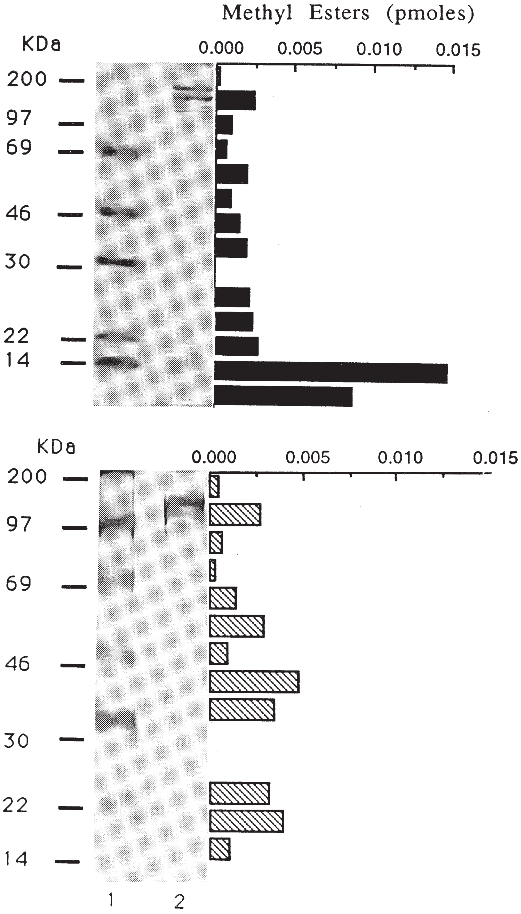

Fig. 2. Comparison of discontinuous acid gel with continuous acid gel. The following experiment tested for the presence of age-altered proteins in a commercial preparation of collagenase. The collagenase preparation (Sigma, type IV) was methylated with purified rabbit erythrocyte PCM and ^{3}H-AdoMet. Aliquots from the same methylation reaction were then resolved on (top) 12% discontinuous acid gel, described in text, or (bottom) 12% continuous acid gel system prepared according to the method of Fairbanks and Auruch *(3)*. *Lane 1*: Rainbow molwt markers (Amersham); *Lane 2*: 18 µg of methylated collagenase were loaded on each gel. Following electrophoresis and staining, 0.5-cm gel slices were treated with base to detect radioactivity as described under **Subheading 3**. Both gel systems are capable of preventing the loss of methyl esters from protein samples, but the discontinuous system provides much higher resolution of individual polypeptide bands.

3. The gels are then placed on a piece of filter paper and dried under vacuum without heat for 3 h.
4. In a dark room, X-ray film (Kodak X-Omat AR) is preflashed twice at a distance of 15 cm with a camera flash unit fitted with white filter paper (3M) to act as a diffuser.
5. The gel is placed in direct contact with the film and taped in place. For future alignment, puncture holes in an asymmetric pattern in a noncrucial area of the gel-film sandwich. A 25-gage needle is useful for this purpose.
6. After sealing in a film cassette, the cassette is wrapped with aluminum foil, and exposure takes place at −70°C for several weeks.
7. After exposure, remove the cassette from −70°C, and allow to warm to room temperature. Develop film in darkroom.
8. An example of this technique is shown in **Fig. 2**.

3.4.2. Scintillation Counting Radioactive Methanol Evolved by Base Hydrolysis of Protein Methyl Esters in Gel Slices

1. Stained gels are soaked in 10% acetic acid containing 3% (v/v) glycerol for 1.0 h, placed on a piece of filter paper, and dried under vacuum without heat for 3.0 h. The glycerol keeps the gel from cracking and keeps it pliable for the steps described below.
2. Using a ruler and fine-tip marking pen, a grid is drawn directly on the surface of the dried gel.
3. A sharp scalpel is then used to cut out uniform slices precisely from each gel lane. Alternatively, selected bands can be individually excised from the gel.
4. 4.0 mL of scintillation fluid are added to each 20-mL scintillation vial. A glass 1-dram vial is then placed inside the scintillation vial, carefully avoiding spilling scintillation fluid into the 1-dram vial.
5. Each dried gel slice is then placed in the inner dram vial of a scintillation vial.
6. After all the gel slices have been placed in a separate inner 1-dram vial, 0.3 mL of 0.2 *M* sodium hydroxide is added to each inner vial. The scintillation vial is then immediately tightly capped and allowed to sit undisturbed for at least 3 h. Several hours are required for any volatile methanol that has formed by methyl ester hydrolysis to equilibrate and partition into the organic scintillation fluid, where it can then be detected by the scintillation counter.
7. Controls for measuring the efficiency of equilibration are performed by using a ^{14}C methanol standard, which is added to an inner 1-dram vial containing a nonradioactive gel slice and base hydrolysis solution. This is then placed inside a scintillation vial and allowed to equilibrate along with the other samples. An equal aliquot of the ^{14}C methanol standard is mixed directly with the scintillation fluid.
8. **Figures 3** and **4** show examples of gels that have been sliced and counted in a scintillation counter under the conditions just described.

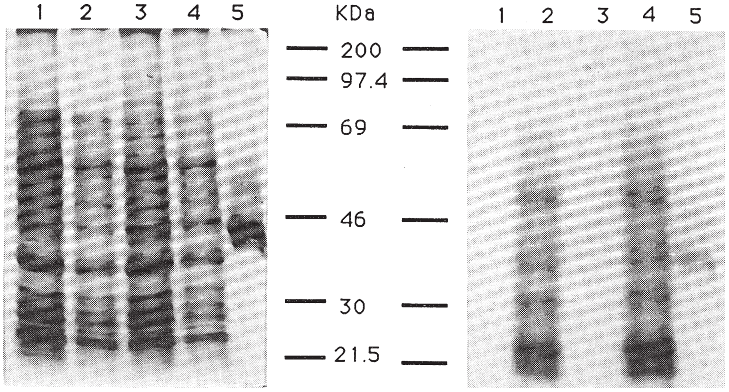

Fig. 3. Coomassie staining and autoradiography of complex protein mixtures by acidic discontinuous SDS gel electrophoresis. The following experiment was performed to measure the varieties of age-altered proteins in a cell cytoplasm. (**Left Panel**) Coomasie G-250 stained acidic discontinuous gel. (**Right Panel**) Autoradiogram of same gel. *Lane 1*: Cytoplasmic proteins, following incubation cells with ^{3}H-*S*-adenosyl-methione (AdoMet). *Lane 2*: Cytoplasmic proteins, following incubation of PC12 cytoplasm with ^{3}H-AdoMet. *Lane 3*: Cytoplasmic proteins, following incubation of intact PC12 cells with ^{3}H-AdoMet and purified rabbit PCM. *Lane 4*: Cytoplasmic proteins, following incubation of lysed PC12 cells with ^{3}H-AdoMet and purified rabbit PCM. *Lane 5*: Positive control; 20 µg of ovalbumin methylated with ^{3}H-AdoMet and purified rabbit PCM. Cells, lysates, and subfraction incubated with IU PCM, 300 pmol Adomet in final volume of 50 µL 0.2 *M* citrate, pH 6.0, 20 min at 37°C.

4. Notes

1. The buffering system employed in the stacking gel and sample solubilization buffer is based on a modification of the C & L buffering system. NaCl is employed rather than KC1 of the original system, because K$^+$ ions cause SDS to precipitate out of solution.
2. On occasion, the solubilization buffer will contain precipitates. These can be brought back into solution by briefly heating the buffer at 37°C. The solubilization buffer is stable for at least 2 wk at room temperature; by storing the buffer in small aliqouts at –20°C, it is stable indefinitely.
3. Gels can be stored for up to two weeks at 4°C by wrapping them in damp paper towels and sealing tightly with plastic wrap.
4. Since the dye front is a poor indicator of protein migration, use of prestained mol-wt markers, such as the colored Rainbow markers from Amersham, allows the progress of protein separation to be monitored by simply identifying the colored bands, which are coded according to molecular weight.
5. It is essential that all fixing and staining steps occur at acidic pH. The colloidal Coomassie G-250 procedure described under **Subheading 3.** has

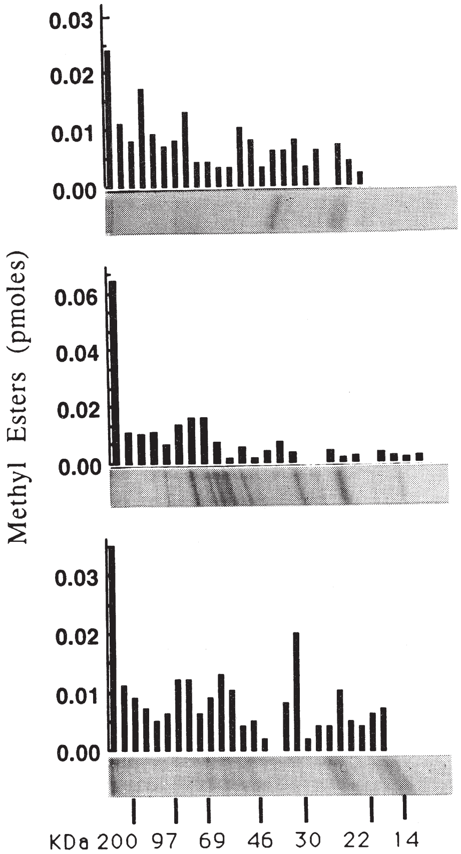

Fig. 4. Coomassie staining and radioactive methyl ester determination in gel slices of electrophoresed proteins from diseased human brain tissue. Extracts prepared from homogenates of Alzheimer's diseased brain (obtained from the Department of Pathology, Oregon Health Sciences University) were methylated in vitro with purified rabbit erythrocyte PCM and ^{3}H-AdoMet. Methylated proteins were then separated on 12% discontinuous, acidic gels, and radioactivity in each gel slice was quantified with scintillation counting as described under **Subheading 3**. *Top*: Distribution of methyl acceptor proteins in tissue protein that was insoluble in the nonionic detergent Triton X-100. *Middle*: Distribution of methyl acceptor proteins in tissue proteins that was soluble in an aqueous homogenization buffer. *Bottom*: Distribution of methyl acceptor proteins in crude homogenates of Alzheimer's diseased brains. Incubation conditions are similar to those described in **Fig. 3.**

several advantages: it is acidic, simple to perform, and has higher sensitivity than other dye-based staining methods, including those using Coomassie R-250. Additionally, nonspecific background staining is very low, so only minimal destaining is necessary to visualize protein bands.

6. Avoid using higher glycerol concentrations or prolonged incubation of the gel in this solution. Otherwise, the gel will be sticky after drying and contract sharply away from the paper backing on cutting.

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Analysis of Tyrosine-*O*-Sulfation

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and Jens F. Rehfeld**

1. Introduction

Tyrosine *O*-sulfation was first described about 50 years ago as a post-translational modification of fibrinogen ***(1)***. In the following thirty years it was considered to be a rare modification affecting only a few proteins and peptides. However, in the beginning of the 1980s tyrosine (Tyr) sulfation was shown to be a common modification and since then an increasing number of proteins have been identified as sulfated. The target proteins belong to the classes of secretory, plasma membrane, and lysosomal proteins, which reflects the intracellular localization of the enzymes catalyzing Tyr sulfation, the tyrosylprotein sulfotransferases (TPSTs). TPSTs are type II integral membrane glycoproteins that reside in the *trans* Golgi network and use adenosine 3′-phosphate 5′-phosphosulfate (PAPS) as a co-substrate and sulfate donor. In higher organisms, 2 enzymes have been identified and cloned and they appear to be ubiquitously expressed in tissues ***(2–4)***. Moreover, homologous genes are present in lower eukaryotes as well as in the nematode *Caenorhabditis elegans*, fruit fly, and zebrafish. Both TPST-1 and TPST-2 knock-out mice models have been established and both display distinct phenotypes. The TPST-1 knock out mice are slightly smaller than wild-type mice and the female produces smaller litters ***(5)***. The TPST-2 knock-out mouse has delayed growth but reaches normal weight at 10 wk. Strikingly, the TPST-2 knock-out male is infertile ***(6)***. The biological function of Tyr sulfation is modulation of protein-protein interactions. For instance, Tyr sulfation is necessary for binding of the peptide hormone cholecystokinin (CCK) to the CCK-1 receptor ***(7)***; it is critical for interactions between p-selectin and PSGL-1 (P-Selectin Glycoprotein Ligand-l) ***(8)***; between hirudin and thrombin ***(9)***; and it modulates the ability of HIV −1 virus to enter cells via the chemokin receptor CCR5 and CD4 ***(10)***. In addition, sulfation has been reported to modulate

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proteolytic processing ***(11)*** and secretion rates of secretory proteins ***(12)***. From these functions, it follows that it is not only important to detect Tyr sulfation, but also to identify the sulfation site to establish effects on the activity on the protein. Attempts have been made to determine a consensus site for tyrosine sulfation and generate algorithms that predict sulfation sites ***(13–15)***, one of these is the "sulfinator," which is available at www.expasy.org. However, it is known that the sulfinator at best gives an indication of possible sulfation sites, and it has been shown that nontypical structures, which are not identified by the sulfinator, can be sulfated ***(16,17)***.

Traditionally, Tyr sulfation has been analyzed by incorporation of radio labelled sulfate into target cells followed by purification of the target protein. Subsequently, the protein is degraded enzymatically or by alkaline hydrolysis followed by thinlayer electrophoresis to demonstrate the presence of radioactively labeled tyrosine. These techniques have been described in detail previously ***(18)***. The aim of this chapter is to present alternative analytical methods of Tyr sulfation other than radioisotope incorporation before analysis. Thus, sulfation of protein or peptide fragments can be demonstrated by a combination of chromatography and specific assays. This has several advantages. First, depending on the assay used, the analysis can be quantitative. Second, problems with incorporation of isotope and the high degree of incorporation of radiolabeled sulfate into carbohydrate moieties that might interfere with detection can be avoided. In addition, it allows two ways of identification of sulfated tyrosines depending on purity of the protein. One strategy demands purified protein using mass spectrometry or chromatography, whereas another uses Tyr sulfate sensitive antisera to distinguish between sulfated and nonsulfated forms in a crude extract. We provide protocols for establishment of sulfation specific antisera and give examples of chromatographic systems that are useful for analysis of Tyr sulfation.

Establishment of a specific immunoassay is costly and time-consuming, and initiation of such a process demands an interest exceeding the mere confirmation of the modification. If such an assay is desirable, a number of considerations have to be taken into account. The size of an antibody binding site is complementary to that of a peptide ligand (or epitope) of 4–6 amino acid residues ***(19)***. Within such an epitope the exact structure of each residue profoundly influences the binding of the antibody ***(20)***. Moreover, the protein or peptide structures surrounding the particular epitope also modulate binding affinity considerably ***(21)***. Accordingly, it is not surprising that attempts to raise antibodies that specifically bind a single *O*-sulfated Tyr residue as such—irrespective of neighboring structures—have failed (Hakanson, R. and Huttner, W. B. personal communications, and our own results). However, recently a promising monoclonal antibody was reported, which was raised against tyrosine sulfated P-selectin glycoprotein ligand-1. It has tyrosine sulfate specificity and show a wide tolerance to sequence variance around the sulfated tyrosine.

Nevertheless, it does not bind sulfotyrosine alone and is thus not entirely sequence independent ***(22)***.

The discrepancy with the success to raise antibodies against the structurally similar phosphorylated Tyr residues ***(23)*** is at present unexplained. In accordance with the aforementioned consideration and with prevailing immunochemical theories, antibodies that recognize sulfation of a given Tyr residue consequently have to be raised against the entire epitope containing the sulfated tyrosine, i.e., a peptide sequence of approx 5 or 6 amino acid residues. It has, however, proven difficult to raise high-titer and high-affinity antibodies or antisera against peptides of less than 10 residues ***(24)***. Therefore, a peptide of approx 10 residues containing the desired epitope in an otherwise expedient sequence should be designed, synthesized, and used for immunization. Hence, measurement of Tyr sulfation by immunochemical methods requires precise knowledge of the sequence and structure surrounding the *O*-sulfated residue in a peptide or protein. It is necessary to consider different strategies in the design of appropriate radioimmunoassays (RIA) (*see* **Note 1**).

Sulfation of a protein or peptide introduces a highly acidic group that alters its local physical and chemical properties such as conformation, hydrophilicity, and pK-value. This can be used to separate sulfated and nonsulfated forms, by various chromatographic techniques, e.g., ion-exchange chromatography and reverse phase high-performance liquid chromatography (RP-HPLC) at near neutral pH under which the conditions for ionization of the sulfate group induces the maximum difference between the two forms (*see* **Note 2**). Also, removal of the sulfate group by digestion with arylsulfatase combined with chromatographic analysis of the peptide before and after the digestion can reveal the presence of Tyr sulfate. Often, identification of sulfated Tyr within a protein requires digestion of the protein into smaller peptides followed by separation of the fragments and isolation of relevant peptide. A number of proteinases may be used, for example trypsin or the endoproteinases Lys-C, Glu-C, and Asp- N, the choice being partly dependent on the individual protein. The details for these techniques are beyond the scope of the present chapter, but may be found in other volumes of this series. These analytical methods can profit from the availability of specific antisera or be carried out using pure proteins.

Having a purified protein offers alternative ways of analysis of Tyr sulfation. The sulfate ester bond is generally considered as labile and particularly susceptible to acidic hydrolysis. Despite of this, the tyrosine sulfate can generally withstand pH in the range of 1–3, often used during many standard analyses such as reversed phase chromatography and mass spectrometry, as long as the temperature is kept at room temperature or below and the exposure time is limited ***(25)***. However, sulfated Tyr can not be identified by protein sequence analysis because of the highly acidic environment and elevated temperatures during the analysis. Instead, nonderivatized Tyr will result. Likewise, the standard acidic hydrolysis used for amino acid analysis is not feasible; instead, demonstration of the presence of intact tyrosin sulfate in a peptide was originally obtained by basic hydrolysis before amino acid analysis ***(26)***. This technique has a limited sensitivity because of an inevitable background. Alternatively, nondestructive digestion can be obtained by aminopeptidase M or a combination of aminopeptidase M and prolidase followed by amino acid analysis ***(27)***. However, these techniques are not used much anymore. Mass spectrometry (MS) is now the preferred tool of analysis of many post-translational modifications. MS in combination with protein sequence analysis can disclose the presence of a sulfated Tyr. Thus, if the identified sequence contains Tyr and the measured mass is 80 Da higher than the calculated value, it is a good indication that the Tyr is sulfated. However, phosphorylation also results in an 80 Da increase of the molecular mass (*see* **Note 3; Fig. 1**). It should also be noted that the sulfate group is lost during ionization in both matrix assisted laser desorption/ionisation (MALDI) and to some degree in electrospray ionization (ESI), resulting in a false negative result. Using MALDI time-of-flight (TOF) MS, the loss of sulfate can be turned into a diagnostic advantage because the loss is often complete in the positive mode ***(31)*** and limited in negative mode. Hence, comparison of spectra obtained in positive and negative linear mode of the same sample will show a vast difference in the yield of the sulfated and nonsulfated species (*see* **Note 4**), whereas the picture will be more complex in the reflector mode (*see* **Note 5; Fig. 2**). In experiments using hybrid instruments of the quadrupole-TOF type it is possible to see fractions of the sulfated species in positive mode even though the desulfated species is generally dominating. The fact that tyrosine sulfate is unstable even at standard collision energy in a quadrupole-TOF instrument, can be used in identifying new sulfated targets by using neutral loss scan experiment detecting the loss of 79.957 ***(32)***. Though tyrosine sulfate and tyrosine phosphate has almost identical mass, it is possible to distinguish between the two as the tyrosine sulfate is generally much more unstable than the tyrosine phosphate, which is not nearly as prone to neutral loss as the tyrosine sulfate. Furthermore when performing MS/MS experiments the tyrosine phosphate gives rise to a stable immonium ion of 216.043 whereas the immonium ion of tyrosine sulfate is not detected. This can be used to further confirm the identification of a phosphate or sulfate. Another option is to investigate tyrosine sulfation by a mass spectrometer capable of electron capture dissociation (ECD). This is a fragmentation method that, to a higher degree, maintains the sulfate during peptide backbone fragmentation but requires specialized and expensive instrumentation ***(33,34)***. Recently, a new method for stoichiometric identification of tyrosine sulfate in proteins using tandem mass spectrometry was described (***35***). The method utilize sulfosuccinimidyl acetate to block unsulfated tyrosines, followed by analysis under conditions that hyrolyze tyrosine sulfate, leaving unmodified

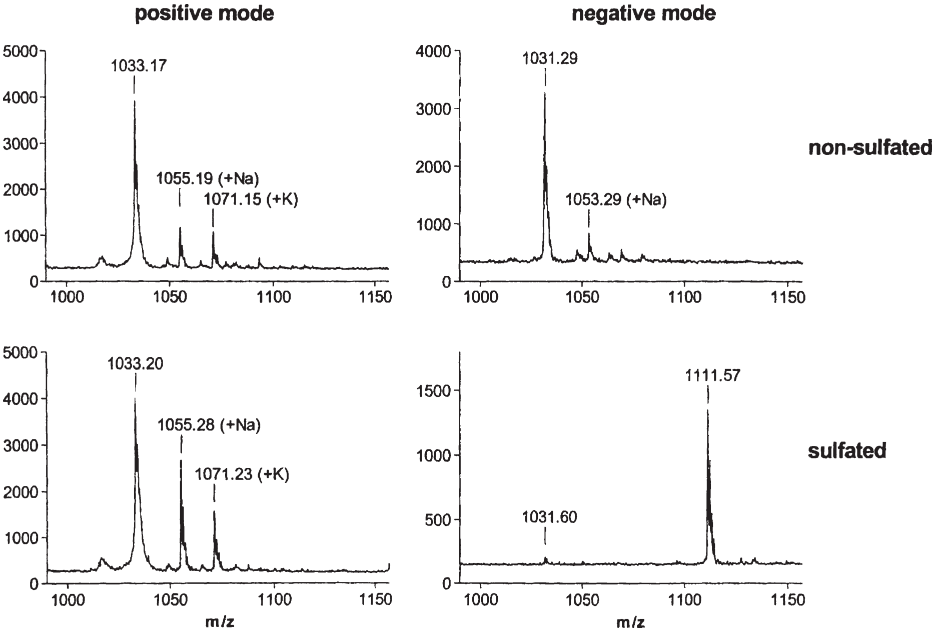

Fig. 1. Mass spectra obtained in the positive and negative mode in a mass spectrometer equipped with delayed extraction (Bruker Daltonik Biflex). As model peptides were used nonsulfated and sulfated shark gastrin-8 (Asp-Tyr(SO_3)-Thr-Gly-Trp-Met-Asp-Phe-NH_2) with the theoretical monoisotopic molecular masses 1032.40 and 1112.36 Da, respectively. In the positive mode the measured *m/z* values represent the protonated molecular ion (+1.01) and to a certain degree the Na^+ and K^+ adducts, whereas the deprotonated form (−1.01) is recorded in the negative mode. In this example the intact sulfated peptide is only observed in the negative mode together with a small fraction of the desulfated form. *See also*, however, **Note 3**. *y*-axis: number of collected ions.

tyrosines as indicators of a previously sulfated site. This method is generally applicable and sensitive, but like other present methods it is not well suited for general screening of sulfated proteins.

2. Materials

2.1. Generation of Tyr Sulfation Sensitive Radioimmunoassays

2.1.1. Preparation of immunogen (common for all Three Coupling Methods) (*see* **Note 6**)

1. 5 mg sulfated peptide.
2. *N, N*-dimethylformamide.
3. 25 mg bovine serum albumin.

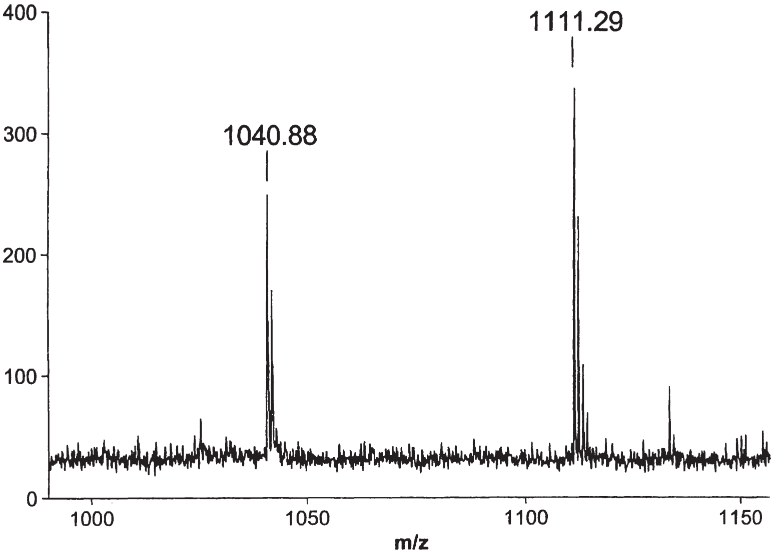

Fig. 2. Mass spectrum of sulfated shark gastrin-8 (*see* **Fig. 1**) obtained in the negative reflected mode using α-cyano-4-hydroxycinnamic acid as matrix. The peak at *m/z* 1111.29 represents the intact peptide (theoretical value 1112.36 −1.01) whereas the peak at 1040.88 represents the in-flight desulfated peptide. The difference between the two is considerably less than the expected 80 (*see* **Note 5**). Note that the resolution is higher in the reflected mode than in the linear mode **(Fig. 1)**.

4. 0.05 *M* sodium phosphate buffer, pH 7.5.

For carbodiimide-coupling:

5. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl.

For glutaraldehyde-coupling:

6. 500 g/L glutaraldehyde.
7. One Sephadex G-10 column with a fraction collector.

For maleimidobenzoyl-succinimede ester-coupling:

8. 25 mg *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS).
9. One Sephadex G-25 column with a fraction collector.

2.1.2. Immunization

1. 6–8 rabbits.
2. 8.5 g/L saline buffer.
3. Freund's complete adjuvant.
4. Freund's incomplete adjuvant.

2.1.3. Preparation of Tracer (*see* **Note 7; Fig. 3**)

1. Synthetic peptide
2. HPLC apparatus with reverse-phase column (Aquapore C-8 column, RP-300, 220 × 4.6 mm, 7-µm bead size) and fraction collector.

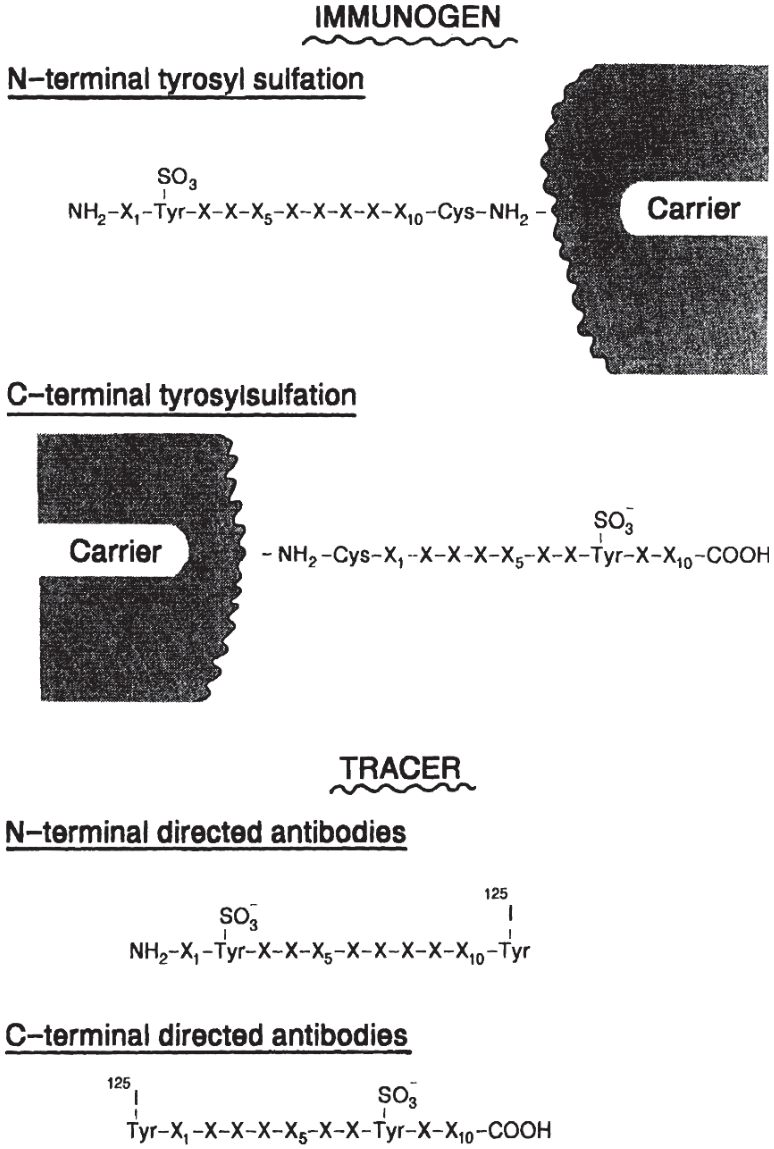
IMMUNOGEN
N–terminal tyrosyl sulfation
SO3
NH2–X1–Tyr–X–X–X5–X–X–X–X–X10–Cys–NH2 –
Carrier
C–terminal tyrosylsulfation
Carrier
SO3-
– NH2–Cys–X1–X–X–X–X5–X–X–Tyr–X–X10–COOH
TRACER
N–terminal directed antibodies
125
SO3-
NH2–X1–Tyr–X–X–X5–X–X–X–X–X10–Tyr
C–terminal directed antibodies
125
SO3-
Tyr–X1–X–X–X–X5–X–X–Tyr–X–X10–COOH

Fig. 3. The structure of immunogens used for production of antibodies directed against tyrosyl *O*-sulfated peptides (upper part of the figure), and the structure of corresponding radioactive tracers (lower part of the figure).

3. Ethanol or acetonitrile (HPLC grade).
4. 1.0 mL/L trifluoroacetic acid (TFA; HPLC grade in water).
5. 0.02 *M* Barbital buffer, pH 8.4.

   Iodination using chloramin T:

6. I^{125} (Amersham IMS 30, specific activity 16.85 mCi/µg of iodine).
7. 0.05 *M* phosphate buffer, pH 7.5.
8. Disodium sulfite (Na_2SO_3).
9. Chloramine T.

   Iodination using Bolton Hunter reagent:
10. Bolton and Hunter reagent for protein iodination (Amersham).
11. 0.2 *M* sodium borate buffer, pH 8.5: mixture of 0.2 *M* $Na_2B_4O_7$ and 0.2 *M* H_3BO_3 with pH adjusted.
12. 0.1 *M* sodium borate buffer with 0.2 *M* glycine, pH 8.5 (prepared from the buffer above).

2.1.4. RIA Procedure

1. Synthetic peptide for standard (*see* **Note 8**).
2. Disposable plastic tubes.
3. 0.02 *M* barbital buffer, pH 8.4, containing 1 g/L bovine serum albumin.
4. Activated charcoal.
5. Blood plasma: this is a mixture of buffer and outdated human plasma from Blood Banks in 0.02 *M* sodium phosphate, pH 7.4. The optimal concentration normally varies between 10–50% plasma.
6. γ-scintillation counter.

2.2. Analysis of Tyr Sulfation Using FPLC Based Ion-Exchange Chromatography (*see* Note 9)

1. An FPLC system equipped with an ion-exchange column, e.g., an 5/5HR MonoQ anion-exchange column (Pharmacia) and a fraction collector.
2. Buffer A: 50 m*M* Tris-HCl, pH 8.2 added 10% (v/v) acetonitrile.
3. Buffer B: 50 m*M* Tris-HCl, pH 8.2 added 10% (v/v) acetonitrile and 1 *M* NaCl.

Both buffers are filtered through a 0.45 µm filter, degassed and stored at 4°C where it is stable for months.

2.3. Analysis of Tyr Sulfation Using Reverse Phase HPLC

1. An HPLC instrument with gradient formation capability and equipped with a suitable RP (reversed phase) column (C8 or C18).
2. Solvents: Stock solution of 0.5 *M* ammonium acetate in water, filtered through 0.45 µm filter (can be kept for several months at 4°C). Solvents A and B are prepared by addition of 20 mL/L of H_2O and acetonitrile, respectively (both HPLC grade), to form 10 *M* ammonium acetate.

2.4. Analysis of Tyr Sulfation Using Mass Spectrometry

1. Instrument: A mass spectrometer for MALDI-TOF MS.
2. Matrix solution: Prepare a 20 mg/mL solution of α-cyano-4-hydroxycinnamic acid in 30% (v/v) acetonitrile/0.1% (v/v) TFA in an Eppendorf tube and centrifuge; use the supernatant as matrix solution. Alternatively 2,5-dihydroxybenzoic acid (DHB)

20 mg/mL in 50% Acetonitrile/1% phosphoric acid can be used (*see* **Note 10**). For larger peptides 3,5-dimethoxy-4-hydroxy-*trans*-cinnamic acid (sinapinic acid) can be used. Premade matrix solutions ("MALDI-grade") are also commercially available (Hewlett-Packard).

3. For neutral loss scanning on quadrupole-TOF instrument Instrument: A hybrid instrument of quadrupole time-TOF type. For complex samples connected to a HPLC instrument equipped with a reversed phased column for separation of peptides. Desalted sample dissolved in 30–50% Acetonitrile 0.2% formic acid for direct infusion, or sample dissolved in aqueous solution for injection on reversed phase column.
4. Solution A: 95% Acetonitrile/0.2% formic acid, solution B: 5% Acetonitrile/0.2% formic acid.

2.5. Arylsulfatase Treatment

1. Arylsulfatase type VIII (Sigma, 20–40 U/mg).
2. Acetate buffer: 0.2 *M* sodium acetate, pH 5.0.
3. 0.2% Sodium chloride.

3. Methods

3.1. Generation of Tyr Sulfation Sensitive Radioimmunoassays

3.1.1. Preparation of the Immunogen

3.1.1.1. CARBODIIMIDE COUPLING

1. Dissolve 5 mg peptide hapten in 1 mL of *N,N*-dimethylformamide.
2. Dissolve 25 mg bovine serum albumin in 2.5 mL of 0.05 *M* sodium phosphate, pH 7.5.
3. Conjugate by the addition of 125 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl to give molar ratios between the peptide, albumin and ethylcarbodiimide of 1:0.1:40. Mix the reagents and incubate for 20 hours at 20°C.
4. Divide the mixture into six portions and store at −20°C until immunization (stable up to one year).

3.1.1.2. GLUTARALDEHYDE COUPLING

1. Dissolve 2.5 mg of sulfated peptide hapten together with 7.5 mg bovine serum albumin in 5 mL of 0.05 *M* sodium phosphate, pH 7.5.
2. Conjugate by dropwise addition of 100 µL of 500 g/L glutaraldehyde.
3. Mix the solution and incubate for 4 h at 20°C.
4. Apply the mixture to a calibrated Sephadex G-10 column and elute at 20°C with 0.05 *M* sodium phosphate, pH 7.5, in fractions of 1 mL.
5. The void volume fractions containing the conjugate are then pooled, divided into 6 portions, and stored at −20°C until immunization (stable for months).

3.1.1.3. MALEIMIDOBENZOYL-SUCCINIMIDE ESTER COUPLING

1. Dissolve 5 mg sulfated peptide hapten together with 25 mg of bovine serum albumin (BSA) in 10 mL 0.05 *M* sodium phosphate buffer (pH 7.5).

2. Dissolve 25 mg of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) in 1 mL *N, N*-dimethylformamide.
3. Conjugate by dropwise addition of the MBS-solution to the sulfated peptide/BSA solution.
4. Stir at room temperature for 30 min.
5. Free MBS is separated from the activated albumin by Sephadex G-25 gel filtration (eluted at 20°C with 0.05 *M* sodium phosphate, pH 7.5, in fractions of 1 mL).
6. Add the sulfated and cysteinylated peptide to the MBS-activated bovine serum albumin and stir the mixture stirred for 3 h at room temperature and a pH of 7.5.

3.1.2. Immunizations

1. The first portion of the antigen is suspended in 8.5 g/L saline to a volume of 5 mL and carefully emulsified with an equal volume of Freund's complete adjuvant. We recommend emulsification by pushing the saline-adjuvant forwards and backwards between two connected 10-mL syringes until a drop of the emulsion remains coherent and condensed on a larger water surface. Randomly bred rabbits are used for immunizations in series of 6–8 rabbits each.
2. Two subcutaneous injections of the mixture are given over the hips in amounts corresponding to 40 µg hapten per animal.
3. Five or more booster injections using Freund's incomplete adjuvant (prepared as described in 1) are then administered simultaneously in all rabbits at 8-wk intervals, using one-half of the initial dose of antigen per immunization.
4. The rabbits are bled from an ear vein 10 d after immunization. Sera from the bleedings are separated and stored at –20°C.
5. The antiserum obtained is evaluated (*see* **Note 11**).

3.1.3. Preparation of Tracer for Radioimmunoassay (*see* **Note 7)**

3.1.3.1. Iodination using chloramine T

1. Dissolve 500 µg peptide in 1 mL 0.05 *M* phosphate buffer, pH 7.5. Aliquot in 10 µL portions and store at –20°C.
2. Dilute 2 mCi I^{125} (~20 µL) with 85 µL 0.05 *M* phosphate buffer, pH 7.5 (final activity about 200 µCi/10 µL).
3. Dissolve 5 mg chloramin T in 10 mL 0.05 *M* phosphate buffer pH 7.5.
4. Dissolve 2 mg disodium sulfite in 10 mL 0.05 *M* phosphate buffer pH 7.5.
5. Mix 10 µL peptide, 10 µL I^{125} and 10 µL chloramin T gently. After 30 s the reaction is arrested with 25 µL disodium sulfite. Shake gently. If more tracer is prepared, do iodination in batches and pool the preparations.
6. Purify the tracer by HPLC. Fractions with maximal counts of reactivity is diluted with 0.02 *M* barbital buffer, pH 8.4 to a final activity of 106 cpm/mL. Store the tracer in 500 µL aliquots at –20°C.

3.1.3.2. Iodination Using the Bolton Hunter Reagent

1. Evaporate the water from the I^{125} Bolton Hunter reagent under a slow flow of nitrogen.
2. Dissolve 5 µg of peptide in 10 µL water and add 10 µL 0.2 *M* sodium borate buffer.

3. Add the peptide solution to the Bolton Hunter reagent and incubate the mixture at 4°C for about 20 h.
4. Add 500 µL 0.1 *M* borate buffer with 0.2 M glycine. Continue directly with HPLC purification of the tracer as described above, or store at 4°C.

3.1.4. RIA Procedure

1. At room temperature, prepare the following mixtures of total volumes of 2.4 mL (RIA is carried out as an equilibrium system at pH 8.4, using 0.02 *M* barbital buffer, pH 8.4, containing 1 g/L bovine serum albumin) in disposable plastic tubes: 2.0 mL of antiserum dilution, 250 µL of tracer solution (giving 1000 cpm, corresponding to 0.5 fmol of freshly prepared ^{125}I-labeled tracer-peptide), and 150 µL of standard solution or sample. All samples should be assayed in duplicate.
2. Incubate at 4°C for 2–5 days to reach equilibrium.
3. Antibody-bound (B) and free (F) tracers are separated by the addition of 0.5 mL of a suspension of 20 mg of activated charcoal and diluted blood plasma to each tube. The tubes are centrifuged for 10 min at 2000 *g*.
4. Count the activities of the supernatant (B) and sedimented charcoal (F) in automatic γ-scintillation counters for 5 min.
5. Calculate the binding percentage as $B - [(B + F) \times D] / (B + F) - [(B + F) \times D] \times 100$, where the "damage" ($D$) is defined as $B \times 100 / (B + F)$ in the absence of antiserum. The damage is usually 2–3%. The peptide concentration is determined from a standard curve based on known standards.

3.2. Analysis of Tyr Sulfation Using FPLC Based Ion-Exchange Chromatography

1. Equilibrate the column to start conditions and inject sample.
2. Elute with an appropriate gradient at the flow of 1 mL/min and collect fractions if necessary.
3. The eluting peptide is monitored by the UV signal recording or any appropriate specific assay. An example of an elution profile examined by radioimmunoassay is given in **Fig. 4**.

3.3. Analysis of Tyr Sulfation Using Reverse Phase HPLC

1. Equilibrate column to start conditions and inject sample.
2. Elute with an appropriate gradient.
3. Record UV signal at 214 nm and—if further analyses are wanted—collect fractions automatically at regular intervals or collect peak manually. An example of an elution profile is given in **Fig. 5**.

3.4. Analysis of Tyr Sulfation Using Mass Spectrometry

3.4.1. MALDI-TOF

1. Samples should be in a final concentration of 0.05–5 pmol/µL, sensitivity depends highly on the nature of the peptide (*see* **Note 12**).

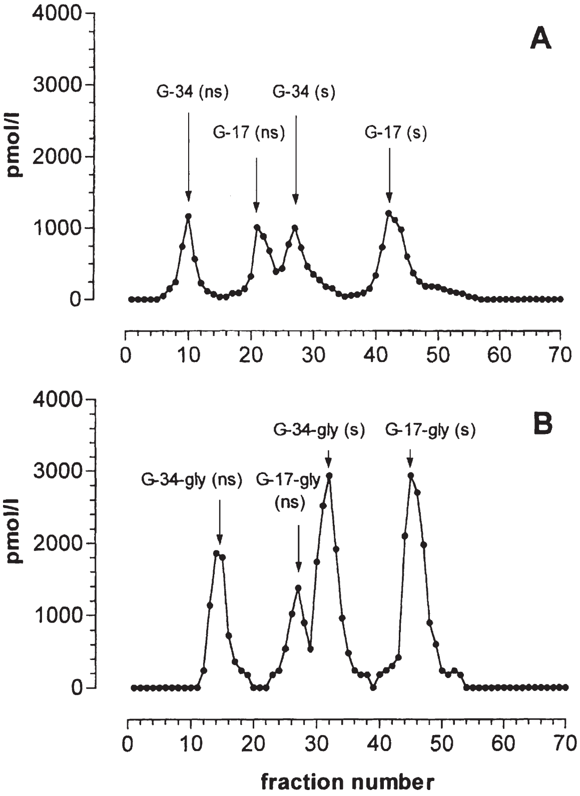

Fig. 4. Anion exchange chromatography using a FPLC system of the main processing forms of human gastrin heterologously expressed in a hamster β-cell line, HIT. **Panel A** shows carboxyamidated gastrin forms of 17 and 34 residues and demonstrates the separation of sulfated and nonsulfated forms. **Panel B** shows the immediate precursor of carboxyamidated gastrin, with the free C-terminus extended with a Gly. Fractions were eluted using a linear gradient of 20–55% buffer B over 60 min at a flow of 1 ml/min. Fractions were analyzed using specific radioimmunoassays. (Elution positions can be verified using synthetic peptides or by analysis of elution positions following arylsulfatase treatment.)

2. Mix 0.5 μL of sample with 0.5 μL MALDI matrix solution. For manual preparation, this can be done in a microcentrifuge tube, or many samples can be prepared consecutively at different positions in a trough made from a cutthrough polypropylene tube.
3. Apply immediately 0.5 μL of the mixture to the sample target (volume may depend on the target size) and let sample dry.
4. Insert sample(s) into the mass spectrometer and record the mass spectrum according to the instrument manual.

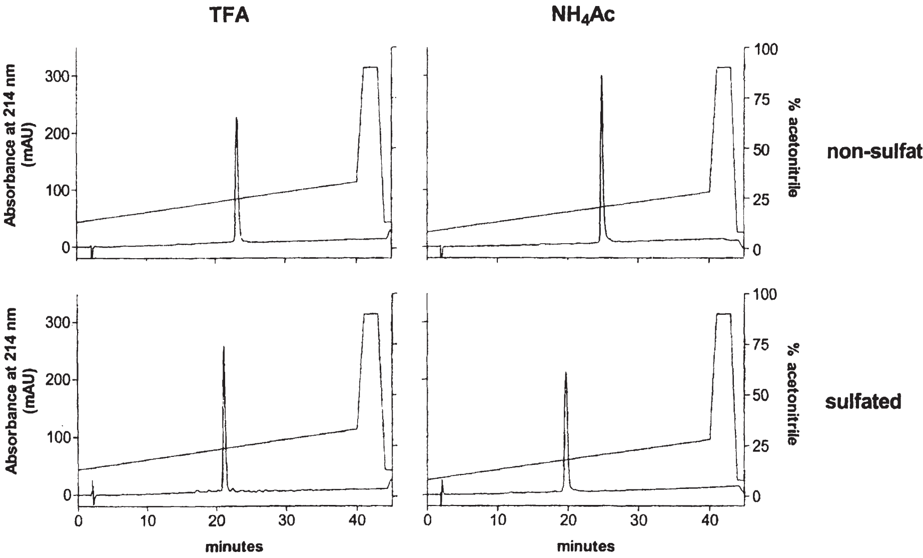

Fig. 5. HPLC separation of the model peptides, non-sulfated and sulfated shark gastrin-8 (*see* **Fig. 1**). The use of 0.1% trifluoroacetic acid (TFA) and 10 m*M* ammonium acetate (NH_4Ac) as buffering component are compared. In both cases 0.5%/min. gradients from H_2O to acetonitrile were employed, starting from 13% acetonitrile in the TFA system and from 8% in the NH_4Ac system (indicated by the straight lines). Although the two forms are indeed separated in the TFA system (by 1% acetonitrile) the separation is much more pronounced in the NH_4Ac system (by 2.5% acetonitrile). For bigger peptides, where sulfation influences the chromatographic behavior relatively less, use of the NH_4Ac system may be crucial for the separation of the non-sulfated and sulfated form.

3.4.2. Neutral Loss Experiment on Quadrupole-TOF: Analyses of complex samples:

1. The LC part of the system is programmed to run a gradient appropriate to the sample, e.g., 5–40% B over 120 min. (*see* **Note 13**).
2. The instrument is programmed to do neutral loss scanning for loss of 79.9568 Da and with alternating slightly higher and slightly lower collision energy than standard settings during the run of the gradient (e.g., on a Q-TOF2 instrument (Waters) collision energies 13 and 8 as opposed to a standard setting of 10), *see* **Fig. 6**.
3. Peptides detected by neutral loss scanning are sequenced by MS/MS (*see* **Note 14**).
4. Tyrosine sulfated peptides are confirmed by the MS/MS data that should not contain the tyrosine phosphate immonium ion of 216.043 Da.

3.4.3. Direct Infusion of Simple Samples

1. For direct infusion, e.g., by nanospray without reversed phase separation the sample has to be clean from salts and only contain a limited number of peptides.

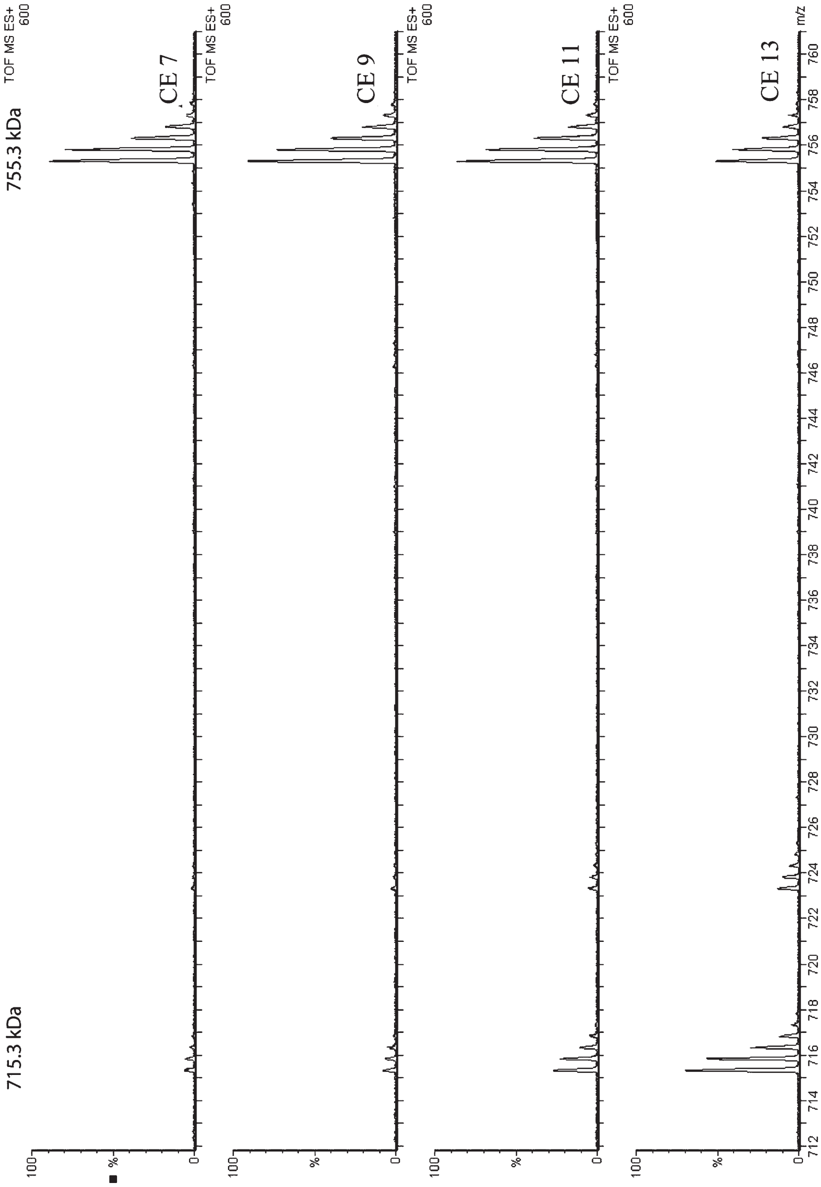

Fig. 6. Mass spectra of tyrosine sulfated drosulfokinin showing the increased neutral loss as the collision energy (CE) is increased in intervals of 2 from 7 to 13. The spectra were obtained on a Q-TOF2 using nanospray ionization. Sulfated drosulfokinin is seen at 755.3 Da and desulfated drosulfokinin at 715.3 Da. Both ions are doubly charged and the neutral loss is therefore observed as 40 Da.

2. The experiment is run as described above but without the LC part. Alternatively collision energy can be changed manually and mass shift for the peptide suspected of being tyrosine sulfated monitored.

3.5. Arylsulfatase Treatment (*see* Note 15)

1. Prepare an enzyme solution of arylsulfatase VIII of 2 mg/mL in 0.2% sodium chloride.
2. Prepare the lyophilized sample by resuspension in 400 µL acetate buffer.
3. Add 100 µL enzyme solution to the sample preparation. The final concentration of arylsulfatase is 0.4 mg/mL (equivalent to 0.55 U/mL).
4. Incubate for 3 h at 37°C.
5. Terminate the reaction by boiling for 10 min.

4. Notes

1. The strategy depends on several factors: (1) The size of the protein or peptide; (2) the position of the sulfated Tyr within the protein; (3) the biology of the sulfated protein or peptide (a large membrane protein; a circulating hormone; or a small neurotransmitter peptide, etc.); and (4) the concentration range in which to measure. If the sulfated Tyr is positioned at the N- or the C-terminus, production of antibodies against a synthetic analogue of the terminally sulfated decapeptide is straightforward. If, however, the sulfated Tyr is located in the middle of a protein or a long polypeptide chain, antibodies should be raised against a fragment containing the sulfated tyrosine, preferably a fragment that can be released from the protein by appropriate proteolytic cleavage. Finally, if the sulfated protein is available in substantial quantities for large immunization series, "shotgun" immunization with the entire protein and subsequent identification of epitope specificity can be attempted.
2. The presence of Met in the peptide presents a possible pitfall, because oxidation of Met to the sulfoxide (which may occur spontaneously in the presence of atmospheric oxygen) increases the hydrophilicity considerably, thus causing the peptide to elute significantly earlier than the reduced form in both a RP-HPLC and in an anion exchange chromatography system.
3. Both sulfation and phosphorylation add 80 Da to the molecular mass of the peptide (the exact monoisotopic values being 79.957 and 79.966, respectively). However, it is possible to distinguish the two by several criteria. Thus, under the standard conditions used for Edman sequencing peptides phosphorylated at Ser, Thr or Tyr give no signal (although for different reasons, ***(28,29)***) resulting in a blank cycle, whereas sulfated Tyr is hydrolyzed and seen as Tyr in almost normal yield. Furthermore, in contrast to Tyr-sulfated peptides **(Fig. 1)**, phosphorylated peptides are recorded as

the intact species in MALDI-TOF linear mode ***(30)***. In the reflector mode a typical triplet is observed from phosphorylated peptides consisting of the intact molecular ion accompanied by a major and a much less abundant fragment because of the loss of H_3PO_4 (98 Da) and HPO_3 (80 Da), respectively ***(30)***. This is in contrast to the singular loss of 80 Da from a Tyr-sulfated peptide (*see* **Note 5**).

4. The relative yields of the sulfated and nonsulfated form depend on the composition of the peptide. Small acidic peptides like the model peptide give almost clean peaks representing the sulfated and nonsulfated form in the negative and positive (linear) mode, respectively **(Fig. 1)**, whereas larger and more neutral peptides give a more mixed picture.
5. A MALDI-TOF mass spectrum obtained in the negative mode using reflector will show the correct molecular ion and an additional peak at a distance *less* than 80 Da below the value for the molecular ion **(Fig. 2)**. This is caused by rupture of the sulfate ester bond in the linear flight tube after the peptide was accelerated but before the peptide reaches the reflector, and the calibration for the reflector requires ions with full accelerating energy. The decrease in molecular mass caused by sulfate loss observed in the reflected mode is instrument dependent, but once established it can also be used as an indicator of a sulfated peptide.
6. The tyrosine 0-sulfated peptide, synthetic or purified has to be available in mg amounts. After dissolution in an appropriate buffer, the peptide is coupled to a protein carrier in amounts that ensure a coupling ratio of approx 5–10 molecule haptens per carrier molecule. There are several useful carrier candidates. We recommend bovine serum albumin (BSA, approx 5 mg/mg hapten decapeptide). BSA is easily available, inexpensive and in our experience as effective as any other protein carrier. When peptide hapten has to be synthesized, we recommend synthesis of an analogue of the genuine hapten equipped with a N- or C-terminal cysteinyl residue. Which terminus or end depends on the position of the sulfated tyrosyl residue **(Fig. 3)**. The peptide hapten is then coupled to a free amino group in the carrier protein through the terminal cysteine using maleimidobenzoic acid N-hydroxysuccinimide ester as coupling reagent ***(36)***. Again, depending on the position of the sulfated tyrosyl residue, conventional coupling using either ethylcarbodiimide or glutaraldehyde may also be used ***(36)***.
7. With directional N- or C-terminal carrier coupling **(Fig. 3)**, it is important to design a tracer that corroborates and further advances the antibody specificity achieved. Because ^{125}I is the preferred RIA-isotope, and because iodine easiest is coupled to tyrosyl- residues, we recommend synthesis of a tyrosylated hapten decapeptide in which the additional tyrosyl-residue for

iodination is placed in the same position as cysteine in the hapten analogue, i.e. at the terminus opposite to the epitopic part of the hapten containing the 0-sulfated tyrosine **(Fig. 3)**. In this way, iodinated tyrosine will not interfere with the antibody binding. There is no risk for iodination of the O-sulfated tyrosine-residue. The sulfonate group blocks incorporation of iodine. Natural occurrence of an additional unsulfated tyrosyl in the short hapten sequence has not been experienced so far; but in such situation a new labeling strategy has to be delineated- based on the exact positions of the additional and the sulfated tyrosyl residue. The tyrosine extended peptide analogue **(Fig. 3)** (4 nmoles) can be monoiodinated using the mild chloramine-T method, previously described ***(37)***. If mild oxidation damages the peptide (containing for instance methionyl residues), it is possible to use the nonoxidative Bolton-Hunter iodination ***(38)***. Subsequent purification on reversed-phase HPLC ensures a high specific radioactivity of the tracer. To evaluate the chromatographic separation of labeled and nonlabeled peptides, 1 ml of the monoiodinated peak fraction is mixed with 10 pmol of the relevant tyrosine-extended peptide and reapplied to the HPLC column as described. Both the radioactivity of the labeled peptides and the immunoreactivity are measured. Using the assumption that iodinated and unlabeled peptides are measured with identical affinity the specific radioactivity of the tracers can be determined by self-displacement ***(39)***. The essence of the described procedure is to ensure monoiodination without oxidative damages to the peptide. High specific radioactivity is then achieved by efficient chromatographic purification.

8. As standard substance for RIA-measurements using antibodies specific for sequence containing a sulfated tyrosyl-residue, it is straightforward to use a peptide corresponding to the hapten decapeptide. If, however, this decapeptide in some way is partly or completely hidden within the structure or conformation of the genuine tyrosyl sulfated protein, or if the antibody requires that epitope has a free N- or C-terminus **(Fig. 3)**, it may be necessary to release the hidden epitope by cleavage with proteases that expose the epitope for antibody binding ***(40)***. If the natural occurring tyrosyl sulfated protein or polypeptide is recognized without cleavage, the protein may as well be used as standard- if available in sufficiently pure form.
9. In this example the peptide analyzed are acidic (the sequence of gastrin-17 is: pQGPWLEEEEEAYGWMDF-NH_2) for which reason an anion exchange column is used. Because Tyr sulfation predominantly occurs in acidic regions of proteins and peptides, similar conditions this will often be appropriate. However, it is also possible to use cation exchange chromatography to separate peptide forms or alternatively, if the net charges of the fragments are neutral, the pH of the buffers may be adjusted.

10. α-cyano-4-hydroxycinnamic acid gives a more homogeneous matrix than DHB and generally also a better signal. However, DHB is a cool matrix compared to α-cyano-4-hydroxycinnamic acid, and hence gives a reduced loss of sulfate in general and especially in reflector mode.
11. Four characteristics of sera from the immunized animals have to be examined (1) The *titer* is defined as the antiserum dilution that binds 33% of the 0.5-fmol tracer at equilibrium. (2) *Affinity* is expressed by the "effective" equilibrium constant (K^0eff), determined as the slope of the curve at zero peptide concentration in a Scatchard plot ***(41)***. (3) *Specificity* is determined in percentage as the molar ratio of the concentrations of the sulfated standard peptide, the unsulfated peptide and other related peptides that produce a 50% inhibition of the binding of the tracer. (4) *Homogeneity* of the antibodies with respect to binding kinetics is expressed by the Sips index ***(42)***. An index of 1 indicates homogeneity of both the tracer and the antiserum in the binding, otherwise seen only for monoclonal antibodies ***(21)***. The ability of the peptides to displace tracers from the antisera may be tested in peptide concentrations of 0, 3, 10, 30, 100, 1000, 10 000, and 100 000 pmol/L.
12. Samples in HPLC buffers are preferable (0.1% TFA in 30% acetonitrile is ideal for MALDITOF). Salt and buffer concentrations should be kept as low as possible. Volatile buffers may be removed in a vacuum centrifuge and the dried sample redissolved in 0.1% TFA/30% acetonitrile. Most detergents, especially SDS, will completely abolish the signal.
13. The gradient should be chosen to give a good separation of peptides. The tyrosine sulfate containing peptides are increasingly suppressed as more peptides are eluted simultaneously. This results in higher risk of missing a tyrosine sulfated peptide in the neutral loss scanning procedure.
14. Note that the sulfate group will be completely lost during MS/MS and the spectrum will show the fragmentation pattern of the desulfated peptide, whereas the precursor ion is that of the sulfated peptide. This constitutes a problem to the general search engines, which are dependent on intact fragment ions of modified peptides to do correct annotation. Therefore manually annotation has to be applied or the parent ion mass must be altered before search.
15. It is our experience that the arylsulfatase treatment is tricky and sometimes very inefficient. We therefore recommend that experiments with negative outcome are repeated and, whenever possible, the inclusion of a positive control peptide with the experiments.

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Analysis of Protein Palmitoylation by Metabolic Radiolabeling Methods

Katherine H. Pedone, Leah S. Bernstein, Maurine E. Linder, and John R. Hepler

1. Introduction

Many eukaryotic signaling proteins are modified by the covalent attachment of long-chain lipids. These highly hydrophobic molecules bind target proteins and facilitate interaction with cellular membranes, lipid molecules and other proteins. Generally, there are three classes of lipids that modify target proteins: myristate, isoprenoids and palmitate (for reviews, *see* ***refs. 1,2***). Myristate, a 14-carbon saturated fatty acid, binds proteins at N-terminal glycine residues via an amide linkage. This usually occurs cotranslationally after removal of the initiating methionine, although it also can occur after proteolytic cleavage and exposure of an internal glycine. Long-chain isoprenoid lipids, including farnesyl and geranylgeranyl groups, modify proteins posttranslationally and attach via a thioether linkage to C-terminal cysteine residues. Palmitate is a 16-carbon saturated fatty acid that modifies proteins posttranslationally through thioester incorporation at cysteines. Palmitate also can modify proteins at additional sites and by alternative mechanisms, as in proteins palmitoylated at serine and threonine residues via oxyester linkages and by amide-linked N-terminal cysteines and glycines. In addition, other fatty acid species form thioester linkages with proteins. For these reasons, the general term for protein lipid modification by thioester attachment is *thioacylation*, or *S-acylation*, and the term *palmitoylation* refers specifically to protein modification by palmitate. Proteins that undergo palmitoylation by thioester attachment at cysteine residues, or *S-palmitoylation*, represent the majority of protein targets of palmitoylation. This chapter will focus on methods for identifying protein substrates for thioester incorporation of palmitate.

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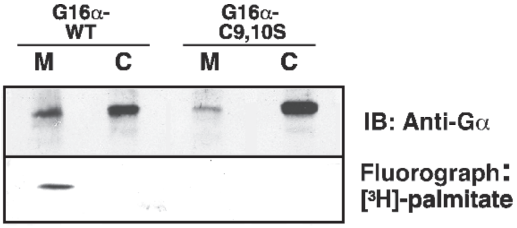
A wide range of proteins become palmitoylated, including intracellular signaling proteins, transmembrane enzymes, secreted morphogens and cell surface receptors. Although the precise role of palmitoylation has proven difficult to discern, the modification has well-described pleiotropic effects on protein function. The common feature shared by all palmitoylated proteins is the capacity for interaction with cellular membranes. The extended hydrocarbon chain of the palmitate group acts as a stable membrane attachment point. This is particularly important for peripheral membrane proteins that otherwise are inherently soluble. The palmitate groups have high affinity for the membrane microdomain structures called lipid rafts in particular, and modification by palmitate is thought to target proteins to these membrane regions for selective protein interactions and signaling purposes. Preventing protein palmitoylation by point mutation or pharmacologic inhibition can exclude proteins from lipid rafts, impair biological activity and has effects on protein-protein interactions, protein stability and subcellular trafficking. For comprehensive reviews of protein palmitoylation, *see* ***refs. 3–5***.

Protein palmitoylation is an enzyme-regulated process that differs from other types of lipid modifications by its reversible nature. Both myristoylation and prenylation are considered stable modifications for the lifetime of the protein, but some palmitoylated proteins exhibit measurable half-lives for the lipid modification. Some intracellular signaling proteins can undergo multiple cycles of depalmitoylation followed by reloading with fresh palmitate. Guanine nucleotide-regulated proteins like Ras and heterotrimeric Gα protein subunits are examples of palmitoylated proteins that dynamically incorporate palmitate during signaling events. The rate of palmitate turnover on these proteins is enhanced dramatically by pharmacologic stimulation of cell surface receptors or direct protein activation (reviewed in ***ref. 6***). For this reason, reversible attachment of palmitate to signaling proteins is thought to be a regulatory mechanism for controlling biological activity of the targeted proteins. Two classes of enzymes that regulate this process have been identified: protein acyltransferases (PATs) catalyze the transfer of palmitate from an intracellular donor such as palmitoyl-coenzyme A to a protein substrate, and protein thioesterases (PTEs) control removal of the lipid. For detailed information on these enzymes and the molecular mechanisms that underlie protein palmitoylation, *see* ***ref. 7***.

The protocols presented in this chapter outline how to assess if a protein is palmitoylated in living cells. The general approach is to incubate cells expressing the protein of interest with [^{3}H]palmitate, isolate the protein from the cells by immunoprecipitation, and use SDS-PAGE and fluorography to determine whether it incorporated radioactive palmitate. In some cases, palmitate may be incorporated into serine or threonine residues through an oxyester linkage, thus it is necessary to verify that a labeled protein is a result of authentic, thioester-linked lipid. This is accomplished by treatment of the gel containing the labeled proteins with neutral hydroxylamine, which hydrolyzes only thioester-linked lipids. This method also is useful for analysis of proteins that have an N-terminal glycine to discern [^{3}H]palmitate incorporation from incorporated [^{3}H]myristate, metabolically converted from the [^{3}H]palmitate. Identity of the attached fatty acid is confirmed by cleaving thioester-linked fatty acids with base. The fatty acids are then extracted from the hydrolysate and analyzed by HPLC or TLC.

The methods described here for palmitoylation analysis by metabolic radiolabeling and fluorography have many advantages. The general experimental techniques are basic and commonly employed in labs utilizing cell culture systems. The specific methods are straightforward and require standard laboratory equipment and few reagents. The primary limitation of this approach is that it only provides a qualitative measure of palmitoylation. The stoichiometry of palmitate incorporation in a protein substrate cannot be determined using these methods. Other methods are available for quantitative analysis of protein palmitoylation. A newly developed technique called fatty acyl exchange labeling is a sensitive and quantitative approach to evaluating protein palmitoylation. In this method, palmitate molecules are cleaved from the modified protein by treatment with neutral hydroxylamine, and the resulting free sulfhydryl groups are coordinated with radiolabeled, thiol-specific reagents like [^{3}H]N-ethylmaleimide (NEM) ***(8)***. This approach also can be adapted for use with non-radioactive (e.g. biotin-conjugated) reagents to bind available cysteine residues. Other methods for analyzing protein palmitoylation include mass spectrometry and in vitro palmitoylation assays with purified and partially purified PATs. These methods are described in more detail with good discussion on advantages and limitations of the techniques, particularly for fatty acyl exchange labeling, in reference ***(8)***.

Once it is established that a protein is palmitoylated, the site(s) for palmitate attachment can be determined by mutation of cysteines to alanine or serine and the mutant protein assayed for loss of palmitoylation. There is no known consensus sequence for palmitate incorporation, so every cysteine on the surface of a protein should be considered a candidate site for palmitoylation. For a review of some shared sequence motifs in palmitoylated proteins, see reference ***(6)***. Proteins that incorporate palmitate at more than one site may contain a primary cysteine, where palmitoylation is required prior to incorporation at other sites within the protein. For this reason, it is important to design both individual and multi-site cysteine mutants to properly map the sites and/or sequence requirements for palmitate incorporation. For our studies, we use the PCR-based QuikChange® Site-Directed Mutagenesis Kit from Stratagene for creating individual and combination cysteine-to-serine point mutations in our proteins of interest (*see* **Fig. 1**). It is important to note that site-directed mutagenesis is an effective way to establish the dependence of the modification on a specific amino acid



G16α: MARSLTWR**CC**PW**C**LTEDEKAAARVDQEINRILLEQKKQDRG

G16α-C9,10S: MARSLTWR**SS**PW**C**LTEDEKAAARVDQEINRILLEQKKQDRG

Fig. 1. Palmitoylation of G16α at N-terminal cysteines determined by metabolic radiolabeling analysis combined with site-directed mutagenesis. Top, upper panel: Immunoblot analysis of G16α-EE wild type (WT) and C9,10S protein immunoprecipitated (anti-EE antibody) from membrane (M) and cytosol (C) fractions of transfected HEK293 cells. Immunoblot analysis was performed with a Gq-family-specific antibody (Z811) kindly provided by Dr. Paul Sternweis (UT Southwestern Medical Center, Dallas, TX). Top, lower panel: Fluorography of G16α-EE WT and C9,10S protein immunoprecipitated from fractionated cells as described above. Exposure time was 56 days at −80°C. Bottom: Sequence alignments of the first 41 amino acids of G16α WT and C9,10S. Cysteine residues for putative palmitoylation and selected cysteine-to-serine mutations are indicated in bold type. Cysteine-to-serine mutations were generated using QuikChange® Site-Directed Mutagenesis kit from Stratagene (cat. no. 200518). (Reproduced from ref. 14 with permission of American Society for Biochemistry and Molecular Biology).

residue. However, definitive proof of site-specific modification requires isolation and identification of the acylated peptide by mass spectrometry.

To successfully perform the techniques described in this chapter, several general considerations should be made. First, specific antibodies are required in order to immunoprecipitate the expressed protein. Since it often is difficult to produce antibodies against proteins directly, epitope-tagged versions of proteins can be engineered. In this chapter, we describe palmitoylation analysis of a G protein, G16α, that contains an internal glutamate-glutamate (EE) epitope tag. Other convenient epitopes can be used instead.

Second, sufficient quantities of protein need to be expressed in order to produce a [^{3}H]-labeled signal. Sf9 insect cells have provided an excellent expression system for palmitoylation studies, particularly for heterotrimeric G protein alpha subunits (Gα) ***(9)***. The primary advantage of insect cells is that infection with recombinant baculovirus produces relatively high levels of heterologous protein expression. Because they are eukaryotic cells, Sf9 contain the cellular enzyme machinery to modify expressed proteins. Detailed protocols for radiolabeling of Sf9 cells and for analysis of lipid modifications can be found in ***ref. 10***.

Although Sf9 cells have proven to be a good model system for palmitoylation studies of Gα proteins, some mammalian proteins may be processed or regulated inappropriately in an insect expression system. Subtle processing differences can have significant effects on palmitate incorporation into the protein. While it is desirable to analyze palmitoylation of any protein in a native system, often proteins are difficult to detect in the endogenous cells or tissues. In this chapter, we describe methods for analyzing palmitoylation of recombinant proteins heterologously expressed in a mammalian cell line. These methods have been used regularly by our labs to evaluate palmitoylation of various G protein subunits and protein Regulators of G Protein Signaling (RGS proteins) ***(9,11–14)***. In this chapter, our example protein is G16α, a G protein alpha subunit from the Gq family of heterotrimeric G proteins. We recently showed that G16α is posttranslationally modified by palmitate and that at least two of three cysteine residues in the N-terminus of the protein are essential for palmitoylation **(Fig. 1)** ***(14)***. We used HEK293 cells for our studies, although COS-7, CHO, or other readily transfectable adherent cell line may be substituted (*see* **Note 1**). Most of the methods described here for mammalian cells are the same or similar to those for Sf9 cells ***(10)***. The differences between the two expression systems are the methods for introduction of the construct into the cells and cell lysis. Since many laboratories are equipped for mammalian cell culture, these protocols are likely to be straightforward to adopt.

2. Materials

2.1. Measuring Palmitate Incorporation into Recombinant EE-Tagged G16α Protein in Mammalian Cell Culture Systems

2.1.1. Transfection and Metabolic Labeling of EE-Tagged G16α in Mammalian Cells

1. HEK293 cells (American Type Culture Collection).
2. Transfection-quality G16α-EE plasmid DNA (*see* **Note 2**).
3. Lipofectamine™ 2000 Transfection Reagent (Invitrogen).
4. Opti-MEM® I reduced-serum media (Invitrogen).
5. Complete HEK293 medium: Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen), 10% fetal bovine serum (Atlanta Biologicals), penicillin/streptomycin (CellGro).
6. [^{35}S]methionine wash medium: 95% methionine-free DMEM (Invitrogen, cat. no. 21013-024), 5% DMEM, 2.5% dialyzed fetal bovine serum (Invitrogen).
7. [^{35}S]methionine labeling medium: 95% methionine-free DMEM, 5% DMEM, 2.5% dialyzed fetal bovine serum, 1 m*M* sodium pyruvate (Invitrogen), 100 μCi/mL L-[^{35}S]methionine (>1000Ci/mmol) (Amersham Biosciences). Aliquot the [^{35}S]methionine and store at −80°C. Make media fresh before use.

8. [^{3}H]palmitate labeling medium: 1 mCi/mL [^{3}H]palmitate ([9,10-^{3}H]-Palmitic Acid (31 Ci/mmol), (PerkinElmer Life and Analytical Sciences), 1% DMSO, 2.5% fetal bovine serum, 1 m*M* sodium pyruvate, 1 m*M* non-essential amino acids (Invitrogen). Make fresh before use (see **Note 3**).
9. Nitrogen gas.

2.1.2. Subcellular Fractionation

1. Potter-Elvehjem tissue grinders with polytetrafluoroethylene (PTFE)-coated pestle, 2 mL capacity, 0.1–0.15 mm clearance (Wheaton Potter-Elvehjem Tissue Grinder, Thermo Fisher Scientific).
2. Ice-cold phosphate-buffered saline (PBS).
3. Hypotonic lysis buffer: 50 m*M* HEPES, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, protease inhibitors (2 µg/mL aprotinin, 1 µg/mL leupeptin, 100 µ*M* phenylmethylsulfonyl fluoride (PMSF)). Add DTT and protease inhibitors just before use.
4. 2X isotonic buffer: 50 m*M* HEPES, pH8.0, 10 m*M* $MgCl_2$, 300 m*M* NaCl, 1 m*M* EDTA, 500 m*M* sucrose, protease inhibitors (4 µg/mL aprotinin, 2 µg/mL leupeptin, 200 µ*M* PMSF). Add protease inhibitors just before use.
5. 1.5 mL capacity microfuge tubes for high-speed centrifugation (Microfuge Tube Polyallomer, Beckman).
6. 2 mL capacity microfuge tubes.
7. 1X Immunoprecipitation (IP) buffer: 50 m*M* HEPES, pH8.0, 5 m*M* $MgCl_2$, 150 m*M* NaCl, 1 m*M* EDTA, 250 m*M* sucrose, protease inhibitors (2 µg/mL aprotinin, 1 µg/mL leupeptin, 100 µ*M* PMSF). Add protease inhibitors just before use.
8. IP buffer with 4% DβM: 4% *n*-dodecyl-β-D-maltoside (DβM; Calbiochem) in 1X IP buffer. DβM is used at a final concentration of 2% for extraction of membrane proteins.

2.1.3. Immunoprecipitation of labeled protein, SDS-PAGE and fluorography

1. Anti-GLU-GLU (EE) monoclonal antibody (Covance).
2. Protein A Sepharose (nProtein A Sepharose® 4 Fast Flow, GE Healthcare).
3. Bovine serum albumin (Sigma).
4. Coomassie blue stain: 30% isopropanol, 10% acetic acid, 0.25% Coomassie brilliant blue R-250.
5. Destain solution: 30% methanol, 10% acetic acid.
6. En^{3}Hance autoradiography enhancer solution (En^{3}Hance™, PerkinElmer Life and Analytical Sciences, cat. no. 6NE9701) (*see* **Note 4**).
7. 2X Laemmli sample buffer: 100 m*M* Tris-HCl pH6.8, 0.5% SDS, 20% glycerol, 0.5% β-mercaptoethanol, 0.004% bromophenol blue. Prepare stocks of 2X Laemmli sample buffer with and without 20 m*M* DTT.

2.2. Hydroxylamine Sensitivity by Thin Layer Chromatography

1. Hydroxylamine solution: 0.5 *M* hydroxylamine hydrochloride (Sigma-Aldrich), pH7.0, in 50% isopropanol. Use concentrated NaOH to pH the solution.
2. 0.5 *M* Tris-HCl, pH7.0, in 50% isopropanol.

2.3. Identification of Fatty Acids on Labeled Protein

1. 13 × 100 mm glass tubes with Teflon-lined caps (Corning, cat. no. 9826-13).
2. HPLC grade methanol, chloroform, acetonitrile, acetic acid.
3. [^{3}H]-fatty acid standards: [^{3}H]palmitate, [^{3}H]myristate, [^{3}H]stearate (Perkin Elmer Life and Analytical Sciences).
4. Nitrogen gas.
5. NaOH.
6. 6N HCl, high grade (Pierce Chemicals).
7. Palmitic acid (Sigma).
8. Trifluoroacetic acid.
9. C:18 reversed-phase thin-layer chromatography plates (Whatman).
10. En^{3}Hance™ Spray Surface Autoradiography Enhancer (Perkin Elmer Life and Analytical Sciences).

3. Methods

3.1. Measuring palmitate incorporation into recombinant G16α-EE protein in mammalian cells

3.1.1. Transfection and metabolic labeling of G16α-EE protein in mammalian cells

This protocol calls for metabolically labeling parallel dishes of transfected cells: one with [^{3}H]palmitate, the counterpart with [^{35}S]methionine. The purpose of labeling cells with [^{35}S]methionine is to control for expression and recovery of the recombinant protein of interest. Alternatively, immunoblot analysis of the protein of interest immunoprecipitated from unlabeled cells may be substituted for radiolabeling with [^{35}S]methionine **(Fig. 1)**.

1. Plate 2–10 cm^2 dishes of HEK293 cells so that they will be 85–90% confluent the next day.
2. Transfect HEK293 cells with G16α-EE DNA construct using Lipofectamine™ 2000 Transfection Reagent according to manufacturer's instructions (*see* **Note 5**). Incubate 5 h, 37°C, 5% CO_2/95% air.
3. Replace transfection media with complete media, and allow cells to recover in 37°C incubator overnight (*see* **Note 6**).
4. Approximately 36 h after beginning the transfection, label one plate with [^{35}S]methionine: wash once in [^{35}S]methionine wash media, then add labeling media. Incubate overnight in the 37°C incubator.

5. The following day, label the second plate with [^{3}H]palmitate: wash cells once with pre-warmed DMEM, then incubate for 1–2 h in [^{3}H]palmitate labeling medium (*see* **Note 7**).
6. Harvest each plate 48 h post-transfection: using a clean razor blade or rubber policeman, scrape cells into separate conical tubes on ice.
7. Collect cells by centrifugation at 500 *g*, 4°C for 4 min.
8. Aspirate media into a receptacle for radioactive liquids. Resuspend cells in 5 mL ice-cold PBS to wash away residual media.
9. Collect cells by centrifugation at 500 *g*, 4°C for 4 min.

3.1.2. Subcellular Fractionation (*see* **Note 8**)

1. Aspirate PBS, and resuspend each cell pellet in 0.5 mL hypotonic lysis buffer. Transfer cells to separate Potter-Elvehjem tissue grinders on ice.
2. Homogenize cells with 30–50 strokes, and transfer lysates to separate 1.5 mL microfuge tubes on ice (*see* **Note 9**).
3. Add 0.5 mL 2X isotonic buffer to each tube to restore normal osmotic pressure.
4. Centrifuge lysates at 600 *g*, 4°C for 10 min to collect nuclei, unbroken cells, and plasma membrane sheets.
5. Transfer the supernatants to Beckman polyallomer microfuge tubes. Spin in TLA100.3 rotor with adaptors, 100,000 *g*, 4°C for 30 min.
6. Remove supernatants from the high-speed spin (cytosol) to clean 2 mL microfuge tubes. Add 1 mL IP buffer with 4% DβM to each for a final detergent concentration of 2% DβM.
7. Resuspend pellets (membranes) in 0.5 mL IP buffer (without detergent) in the polyallomer tubes, and transfer to Potter-Elvehjem tissue grinders on ice. Lightly homogenize the membrane pellets (15 strokes).
8. Add 0.5 mL IP buffer with 4% DβM to each membrane sample for a final detergent concentration of 2% DβM.
9. Solublize membranes by incubating at 4°C for 3 h on an end-over-end rocker (*see* **Note 10**). Incubate cytosol samples similarly for the same duration.
10. Pellet the insoluble material from membrane samples by high-speed centrifugation at 100,000 *g*, 4°C for 30 min.
11. Remove the supernatants (membrane extract) to clean microfuge tubes (*see* **Note 11**).

3.1.3. Immunoprecipitation of Labeled Protein, SDS-PAGE, and Fluorography (*see* **Note 12**)

1. Preblock membrane extracts and cytosol samples in 0.7 mg/mL BSA solution and pre-absorb with 50 µL Protein A Sepharose. Incubate with end-over-end rotation, 4°C from 30 min to 2 h.
2. Add 6–7 µL anti-EE antibody to each sample of membrane extracts and cytosol. Incubate at 4°C at least 3 h, or overnight if convenient, rotating end-over-end.
3. Collect sepharose beads and immunoprecipitated protein complexes by centrifugation: 200 *g*, 4°C for 1 min.
4. Aspirate supernatant, and resuspend sepharose beads in 1 mL IP buffer containing 0.2% DβM.

5. Repeat spin: 200 *g*, 4°C for 1 min. Wash sepharose beads two more times in the same fashion.
6. Suspend the washed beads in 60 µL 2X Laemmli sample buffer without DTT (*see* **Note 13**).
7. Boil samples 1 min, then spin 1 min in microcentrifuge at maximal speed. Load the supernatant samples on SDS-PAGE gels and run (*see* **Note 14**).
8. Stain gels with Coomassie blue stain, rocking, 30 min.
9. Incubate in several changes of destain solution, rocking, until proteins are visualized on the gel (*see* **Note 15**).
10. Soak gels in En^{3}Hance™ autoradiography enhancer solution, 60 min, with gentle rocking.
11. Wash gels thoroughly in a large volume of cold water for 30 min with gentle rocking.
12. Dry down the gels to completion on a vacuum dryer, 60°C.
13. Expose the gels to film in a cassette. Store the cassette at −80°C for at least 3 days to visualize the [^{35}S]-labeled bands. Replace with new film and expose for 1–4 weeks to visualize [^{3}H]-labeled bands (*see* **Note 16**).

3.2. Hydroxylamine Sensitivity of [^{3}H]palmitate-Labeled Proteins (see Note 17; Fig. 2)

1. Transfect cells, label and fractionate cells as in **steps 3.1.1** and **3.1.2** (*see* **Note 18**).
2. Immunoprecipitate EE-tagged protein from cells as outlined in 3.1.3, steps 1–6 above.
3. Boil samples 1 min, then spin 1 min in microcentrifuge at maximal speed. Divide each sample in half, and run duplicate gels of each.
4. Fix gels by incubating in destain solution for 30 min, rocking.

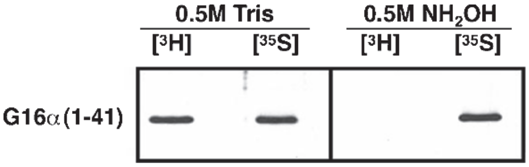


Fig. 2. Confirmation of thioester linkage of [3H]palmitate by hydroxylamine cleavage. Fluorography of the radiolabeled G16α N-terminus with and without neutral hydroxylamine treatment. A peptide representing the N-terminal domain of G16α (amino acids 1–41) was expressed in HEK$_{293}$ cells as a FLAG-tagged GFP fusion protein. The fusion protein was immunoprecipitated (Anti-FLAG M2 Affinity Gel, Sigma, cat. no. A$_{2220}$) from transfected membranes of HEK$_{293}$ cells metabolically radiolabeled with either [^{35}S]methionine or [^{3}H] palmitate. Immunoprecipitated proteins were treated with 0.5 M Tris, pH7.0 (left panel) or 0.5 M hydroxylamine, pH7.0 (NH$_2$OH, right panel) and prepared for fluorographic analysis. Exposure time was 2 days at −80°C for [^{35}S]-signals, 5 days for [^{3}H]-signals. (Reproduced from ***ref. 14*** with permission of American Society for Biochemistry and Molecular Biology.)

5. Soak one gel in fresh 0.5 *M* hydroxylamine solution (pH7.0) for 12–18 hours (overnight). As a control, soak the other gel in 0.5 *M* Tris (pH7.0) in 50% isopropanol. Replace the solutions at least once after approximately one h. The gels will shrink as they are dehydrated by the isopropanol.
6. Wash each gel 4 times in 50% isopropanol over a period of 48 h.
7. Soak gels in Coomassie stain, then destain solutions as above to visualize precipitated proteins. The gels will rehydrate and return to original size during this step.
8. Soak gels in En^{3}Hance™ autoradiography enhancer solution for 60 min with gentle rocking.
9. Dry down the gels on a vacuum dryer, 60°C, and expose to film at −80°C.

3.3. Identification of Fatty Acids on Labeled Protein

3.3.1. Base Hydrolysis and Extraction of Thioester-Linked Fatty Acids

1. Transfect cells, label, and fractionate cells as in **steps 3.1.1** and **3.1.2**.
2. Immunoprecipitate protein from cells as outlined in **Subheading 3.1.3, steps 1–6**.
3. Boil samples 1 min, then pellet for 1 min in microcentrifuge at maximal speed. Divide each sample in half and run duplicate SDS-PAGE gels.
4. Stain gels with Coomassie blue stain, rocking, 30 min.
5. Incubate in several changes of destain solution until proteins are visualized on the gel.
6. Excise the immunoprecipitated protein bands from membranes and cytosol with a razor blade or scalpel, keeping gel slices equal in size. Also excise three gel slices from unused portions of the gel to run with standards.
7. Place gel slices in air-tight, screw-cap 13 × 100 mm glass tubes. Wash gel slices overnight with five changes of 50% methanol.
8. Remove methanol with Pasteur pipet. To the blank gel slices, add [^{3}H]myristate, [^{3}H]stearate, or [^{3}H]palmitate standards, 25,000–50,000 dpm per sample. Dry gel slices under a nitrogen stream.
9. To hydrolyze thioester-linked lipids, resuspend gel slices in 0.7 mL 1.5 *M* NaOH. Flush tubes with nitrogen and cap. Incubate 3 h, 37°C, mixing periodically.
10. Acidify the solutions to pH1-2 by adding 6N (high-grade) HCl. This typically requires 100–200 µL of acid.
11. To extract the released fatty acids, add 3.75 volumes of chloroform:methanol (1:2 v/v) in a fume hood. This results in chloroform:methanol:aqueous 1.25:2.5:1 v/v/v. Incubate 10 min, room temperature with occasional mixing.
12. To separate the fatty acids from water-soluble components and gel slices, add 1.25 volumes (of original aqueous volume) of chloroform and 1.25 volumes of water.
13. Vortex tubes, then centrifuge at 1,000 rpm at room temperature. This will separate the mixture into an upper aqueous phase and a lower organic phase, with the gel slice in between.
14. Remove the bottom phase containing fatty acids by bubbling a Pasteur pipette through the aqueous phase to the bottom of the tube. Transfer to new screw-cap tubes.
15. To extract remaining fatty acids, add the same amount of chloroform as in **step 12**. Incubate 5 min, room temperature with occasional mixing. Centrifuge 1,000 rpm and remove bottom phase as before. Combine with first extracts.

16. Dry down extracts under nitrogen.
17. Add 0.5 mL chloroform and 40 μg cold palmitic acid as a carrier. Flush tubes with nitrogen and store at −20°C.

3.3.2. Thin-Layer Chromatographic Analysis of Hydrolyzed Lipids

1. Count a small volume of each sample (50 μL) in scintillation vials to determine how much sample to load. Let the chloroform dry before counting or it will quench.
2. Spot equivalent amounts of each sample (25,000–50,000 dpm) on a reverse-phase C:18 TLC plate. Also spot equivalent standards.
3. Resolve the extracts using 90% acetonitrile/10% acetic acid in a chromatography tank. This should take 20–30 min.
4. After the solvent has migrated to the top, remove plates and allow them to dry thoroughly in the hood.
5. Spray plates with En^{3}Hance™ spray. Let plates dry slightly and expose to film at −80°C for 5–10 h. Re-expose for 24–48 h if signal is undetectable.

4. Notes

1. The protocols for heterologous protein expression, cell lysis and fractionation (**Subheadings 3.1.1** and **3.1.2**) are described here specifically for mammalian cells. The remainder of the methods are performed the same independent of eukaryotic cell type used.
2. We use Midi- or Maxi-prep kits from Qiagen to produce DNA for transfections. Follow the manufacturer's protocols. Our G16α-EE DNA plasmid came from UMR cDNA Resource Center (www.cdna.org; cat. no. GNA1500000). Many other DNA constructs for mediators of G protein signaling (GPCRs, G proteins, RGS proteins) also are available from UMR in epitope-tagged and untagged forms.
3. [^{3}H]palmitate is supplied as a solution in ethanol and cannot be added directly to media. First, measure the appropriate amount into a glass tube and evaporate the ethanol under a nitrogen stream. When the palmitate is dry, it will appear as a light film on the bottom of the tube. Resuspend the palmitate first in DMSO (for final concentration of 1% in palmitate labeling media; volume will depend on final volume of labeling media), then add the fetal bovine serum (final concentration 2.5%). Add the remaining reagents to the dissolved [^{3}H]palmitate.
4. We have successfully used a homemade version of fluorographic reagent: 1 *M* sodium salicylate in 15% methanol. Store in a dark bottle.
5. Detailed instructions for optimizing transfection conditions with Lipofectamine™ 2000 Transfection Reagent are provided by the manufacturer. The amount of DNA and lipofectamine used per dish can vary depending on the protein to be expressed.

6. The optimal time to harvest transfected cells for peak protein expression may vary depending on the protein. In this protocol, cells are collected 48 h post-transfection. For shorter post-transfection incubations, labeling should be started at the appropriate time so that [^{35}S]methionine labeling media is incubated overnight and [^{3}H]palmitate media is incubated for 1–2 h.
7. Some proteins undergo turnover of the palmitate modification. For example, the half-life for palmitate on H-Ras has been measured as approximately 2.4 h; however, the half-life decreases to 90 min for ectopically expressed, activated H-Ras, suggesting that G protein activation is linked to palmitate turnover ***(15)***. The propensity for palmitate turnover should be considered when establishing a time period for metabolic labeling with [^{3}H]palmitate. A 1–2 h labeling period with high concentrations of [^{3}H]palmitate as suggested in this protocol is a good starting point. G protein alpha subunit palmitoylation has been detected with labeling periods as short as 15 min ***(16)***.
8. All solutions should be kept cold (4°C), and all samples should be kept on ice during the following steps in order to minimize protein degradation.
9. If using the same Potter-Elvehjem tissue grinder for both [^{35}S]- and [^{3}H]-labeled cells during an experiment, pass the [^{3}H]-labeled samples first to avoid accidental transfer of the higher energy [^{35}S]-signal into the [^{3}H]-labeled samples. Wash out the tissue grinder thoroughly with ethanol to remove trace [^{3}H]-residue. Alternatively, cells may be lysed by another convenient method (e.g. repeated cycles of snap-freezing and quick-thawing).
10. Other buffers may be used to solublize proteins successfully. We found this HEPES-based buffer formulation with 2% DβM detergent to work well for extraction and immunoprecipitation of our Gα proteins with the anti-EE antibody. If a different antibody is used, the buffer may need to be adjusted. Also, a shorter incubation period for membrane extraction may be sufficient for other proteins. Generally, a 1-h extraction period is sufficient to release proteins from membranes prepared at 1 mg/mL membrane protein concentration.
11. At this step, the membrane extracts and cytosol samples may be snap-frozen in liquid nitrogen and stored at −80°C until later use, as limited by the half-life of the [^{35}S]-signal. Frozen samples should be thawed quickly and kept on ice.
12. Immunoprecipitation protocols vary widely. For a review and guide to optimization of immunoprecipitation using different types of antibodies and solid support matrices, see reference ***(17)***. Other protocols may be easily substituted.
13. The thioester linkage for S-palmitoylation is vulnerable to reducing agents such as DTT. For this reason, we exclude DTT from our Laemmli sample buffer for [^{3}H]palmitate-labeled samples. This does not affect migration

of our G proteins on an SDS-polyacrylamide gel, but the same may not be true for other proteins. 20 m*M* DTT may be included in [^{3}H]palmitate-labeled samples if needed, but the lability of the thioester linkage should be considered. Laemmli sample buffer containing 20 m*M* DTT should be used for all other protein samples prepared for SDS-PAGE.

14. Avoid loading sepharose beads onto the SDS-PAGE gel. Thioester-linked fatty acid also is susceptible to prolonged heating. We have found that excluding DTT from the sample buffer and heating for 1 min is sufficient to obtain good resolution of Gα proteins on gels without a substantial loss of radioactive palmitate on the protein.
15. The gel can be left in destain solution overnight if desired.
16. Do not use plastic wrap to cover the gel during exposure as it will block the [^{3}H]-signal. Storage of the cassette at −80°C is essential for the En^{3}Hance™ fluorography reagent to work. Do not use a screen. The [^{35}S]-labeled samples may be visualized within a few days. The [^{3}H]-labeled bands may be detected after one week exposure, but it can take one month or longer, depending on the amount of protein on the gel. Exposure time must be determined empirically.
17. If the membrane-bound protein of interest incorporates [^{3}H]palmitate, further analysis should be done to confirm authentic thioester palmitate attachment. Treatment of the gel containing the [^{3}H]-labeled sample with neutral hydroxylamine should result in the reduction or disappearance of the labeled band. As a control, a duplicate gel is soaked in Tris buffer at the same pH. The [^{3}H]-labeled sample should be visible in this gel. Finally, [^{35}S]methionine-labeled proteins should be run in parallel to control for the loss of protein from the hydroxylamine- and Tris-treated gels during processing. A hydroxylamine-insensitive label can be analyzed as a potential amide-linkage by treating the gels with acid instead. Amide-linked acids are insensitive to hydroxylamine treatment, but are sensitive to acid hydrolysis.
18. The number of cells required for these experiments may vary depending on protein expression and stoichiometry of palmitoylation. Adjust the size and/or number of dishes transfected based on the autoradiograph in **Subheading 3.1**.

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Incorporation of Radiolabeled Prenyl Alcohols and Their Analogs into Mammalian Cell Proteins

A Useful Tool for Studying Protein Prenylation

Alberto Corsini, Christopher C. Farnsworth, Paul McGeady, Michael H. Gelb, and John A. Glomset

1. Introduction

Prenylated proteins comprise a diverse family of proteins that are posttranslationally modified by either a farnesyl group or one or more geranylgeranyl groups ***(1–3)***. Recent studies suggest that members of this family are involved in a number of cellular processes, including cell signaling ***(4–6)***, differentiation ***(7–9)***, proliferation ***(10–12)***, cytoskeletal dynamics ***(13–15)***, and endocytic and exocytic transport ***(4,16,17)***. The authors' studies have focused on the role of prenylated proteins in the cell cycle ***(18)***. Exposure of cultured cells to competitive inhibitors (statins) of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase not only blocks the biosynthesis of mevalonic acid (MVA), the biosynthetic precursor of both farnesyl and geranylgeranyl groups, but pleiotropically inhibits DNA replication and cell-cycle progression ***(10,18–20)***. Both phenomena can be prevented by the addition of exogenous MVA ***(10,18,19)***. The authors have observed that all-*trans*-geranylgeraniol (GGOH) and, in a few cases, all-*trans*-farnesol (FOH) can prevent the statin-induced inhibition of DNA synthesis ***(21)***. In an effort to understand the biochemical basis of these effects, the authors have developed methods for the labeling and two-dimensional gel analysis of prenylated proteins that should be widely applicable.

Because of the relative diversity of prenylated proteins, it is important to use analytical methods that differentiate between them. A useful approach discussed here is to selectively label farnesylated or geranylgeranylated proteins using [^{3}H] labeled FOH or GGOH ***(22–24)***, followed by one-dimensional

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SDS-PAGE or high-resolution twodimensional gel electrophoresis (2DE) of the labeled proteins. Since the enzymes that transfer prenyl groups to proteins utilize the corresponding prenyl alcohol pyrophosphate (FPP or GGPP) as substrate, these prenols are thought to undergo two phosphorylation steps prior to their subsequent utilization ***(2,3)***. The discovery of a GGOH kinase and a geranylgeranyl phosphate kinase in eubacteria ***(25)***, together with the ability of rat liver microsomal and peroxisomal fractions to form FPP, provide additional evidence that these prenol pools serve as a source of lipid precursor for protein prenylation. When proteins are labeled in this way, and are subsequently analyzed by one-dimensional SDS-PAGE, it is possible to distinguish several major bands of radioactivity that correspond to two apparently distinct subsets of proteins: those that incorporate [^{3}H]-FOH and those that incorporate [^{3}H]-GGOH. But when the labeled proteins are analyzed by high-resolution 2DE, the number of radioactive proteins that are observed is at least fivefold greater, and three subsets of prenylated proteins can be identified: one subset of proteins that incorporates only farnesol, a second that incorporates only geranylgeraniol, and a third that can incorporate either prenol.

In this chapter, the advantages of using labeled prenols to dissect the differential effects of FOH and GGOH on cellular function will be presented in the context of studies of the role of prenylated proteins in cell-cycle progression. The ability of mammalian cells to incorporate natural and synthetic prenol analogs **(Fig. 1)** into specific proteins also will be discussed. In **Subheading 4,** some of the advantages and limitations of the methods will be discussed.

2. Materials

2.1. Prenols and Analogs: Synthesis and Labeling

1. All-*trans*-FOH, d^{20} 0.89 g/mL; mevalonic acid lactone; geraniol, d^{20} 0.89 g/mL (Sigma, St. Louis, MO).
2. Tetrahydrofarnesol was a gift from Hoffman la Roche (Basel, Switzerland). A racemic form can be made from farnesyl acetone following the procedure for hexahydro-GGOH (*see* **Subheading 3.1.1.**).
3. All-*trans*-GGOH, d^{20} 0.89 g/mL; all-*trans*-GGOH [1-^{3}H], 50–60 Ci/mmol; all-*trans*-FOH [1-^{3}H], 15–20 Ci/mmol; geraniol [1-^{3}H], 15–20 Ci/mmol (American Radiolabeled Chemicals, St. Louis, MO).
4. Mevalonolactone RS-[5-^{3}H(N)], 35.00 Ci/mmol (NEN, Boston, MA).
5. Charcoal; $LiAlH_4$ powder; MnO_2; $NaBH_4$ powder; triethyl phosphonoacetate; phosphonoacetone; sodium ethoxide (Aldrich, Milwaukee, WI).
6. [^{3}H]-$NaBH_4$ solid, 70 Ci/mmol (Amersham, Arlington Heights, IL).
7. Silica gel 60 (F254) (Merck, Gibbstown, NJ).
8. Aquamix liquid scintillation solution (ICN Radiochemicals, Irvine, CA).

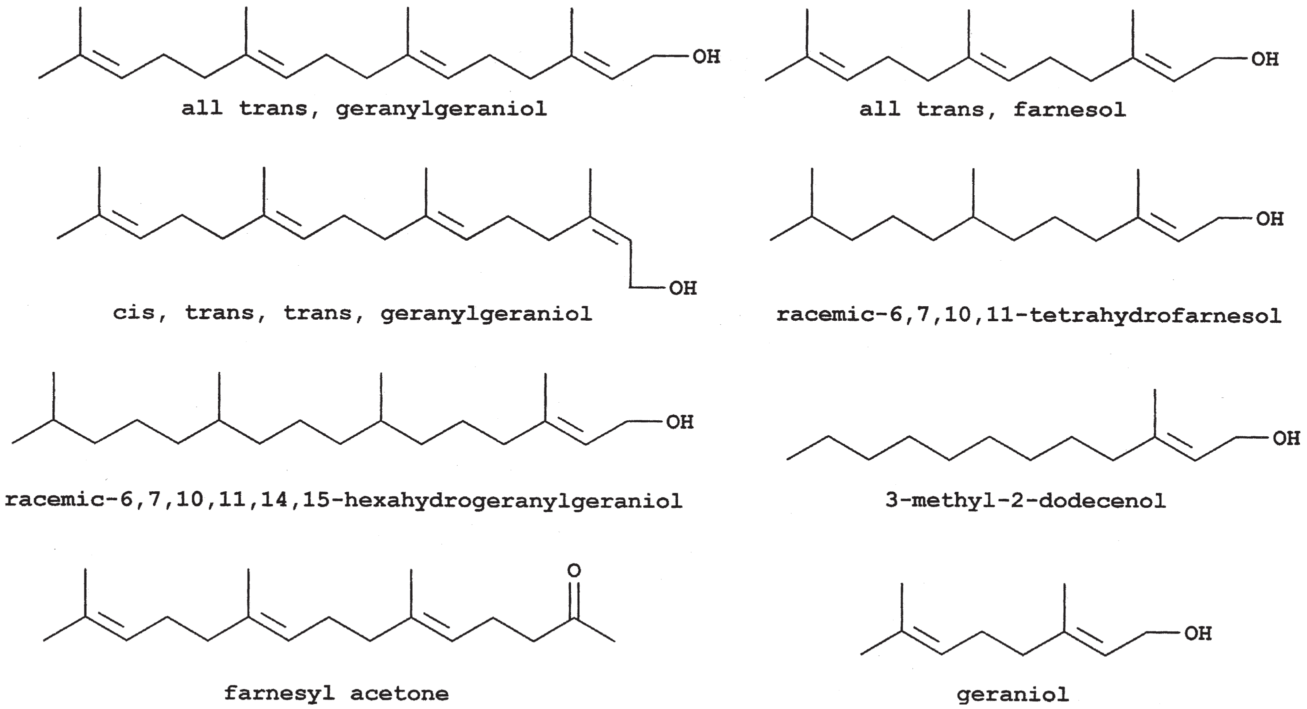
OH
all trans, geranylgeraniol
OH
all trans, farnesol
OH
cis, trans, trans, geranylgeraniol
OH
racemic-6,7,10,11-tetrahydrofarnesol
OH
racemic-6,7,10,11,14,15-hexahydrogeranylgeraniol
OH
3-methyl-2-dodecenol
O
farnesyl acetone
OH
geraniol

Fig. 1. Structure of the natural and synthetic prenol analogs used in these labeling studies.

2.2. Cell Culture Reagents

1. Dulbecco's modified Eagle media (DMEM) (glucose 4.5 g/L); penicillin (10,000 U/mL)–streptomycin (10 mg/mL); trypsin (0.25% w/v)–1 m*M* ethylenediaminetetraacetate (EDTA); nonessential amino acids (NEAA) solution 10 m*M*; phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}; fetal calf serum (FCS) (Gibco, Grand Island, NY).
2. Disposable culture Petri dishes (100 × 10 mm and 35 × 10 mm) (Corning Glass Works, Corning, NY).
3. Filters, 0.22 µm (Millipore, Bedford, MA).
4. Plasma-derived, bovine serum (PDS) (Irvine Scientific, Santa Ana, CA).
5. Aquasol scintillation cocktail; thymidine [methyl-^{3}H], 2 Ci/mmol] (NEN).
6. Trichloroacetic acid (TCA) (J. T. Baker, Phillipsburg, NJ).
7. Phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, and pepstatin A (Sigma).
8. Simvastatin in its lactone form (gift from Merck, Sharp and Dohme; Rahway, NJ) is dissolved in 0.1 *M* NaOH (60°C, 3 h), to give the active form. Adjust the pH to 7.4 and the concentration to 50 m*M*, then sterilize by filtration.
9. Swiss 3T3-albino mouse cell line (3T3) were from American Type Culture Collection (ATCC), Rockville, MD.
10. Human skin fibroblasts (HSF) are grown from explants of skin biopsies obtained from healthy individuals. The cells are used between the fifth and fifteenth passages.

2.3. One-Dimensional and Two-Dimensional Gel Electrophoresis

1. SDS, TEMED, ammonium persulfate, mol wt protein standards, glycine, Bradford protein assay kit, heavyweight filter paper, and Bio Gel P6-DG (Bio-Rad, Hercules, CA).
2. Duracryl™ preblended acrylamide solution (30% T, 0.65% C, used for both one-dimensional and 2DE gels) and all other 2DE gel reagents, were obtained from ESA, Chelmsford, MA.
3. Ethylmaleimide, N-[ethyl-1,2-^{3}H] ([^{3}H]-NEM), 57 Ci/mmol (NEN).
4. N-ethylmaleimide (Aldrich).
5. Soybean trypsin inhibitor and SDS-7 mol wt standards used with 2DE gels (Sigma).
6. Amplify and Hyperfilm (Amersham).
7. Reacti-Vials™ (Pierce, Rockford, IL).
8. DNaseI and RNaseA (Worthington Biochem, Freehold, NJ).

3. Methods

3.1. Synthesis and Labeling of Prenols and Analogs

3.1.1. Synthesis of Racemic 6,7,10,11,14,15 Hexahydrogeranylgeraniol

1. This synthesis is outlined in **Fig. 2**. Weigh 10.4 g of farnesyl acetone and 500 mg 10% Pd on charcoal, dissolve in 100 mL methanol and hydrogenate at 50 psi in a Parr

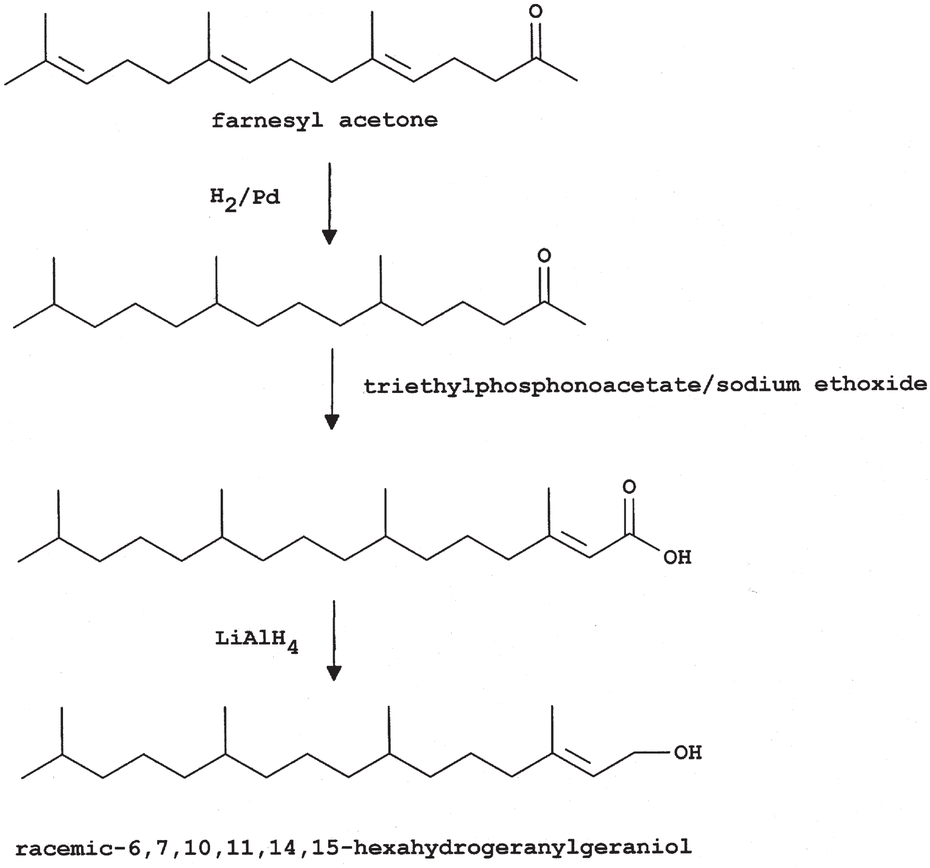
farnesyl acetone
H2/Pd
triethylphosphonoacetate/sodium ethoxide
OH
LiAlH4
OH
racemic-6,7,10,11,14,15-hexahydrogeranylgeraniol

Fig. 2. Schematic for the synthesis of racemic 6,7,10,11,14,15 hexahydro-geranylgeraniol from farnesyl acetone.

hydrogenator for 2 d. Analysis by TLC (7:3 hexanes/diethyl ether) and GC-MS should indicate that the material is fully converted to the hexahydrofarnesyl acetone.

2. Filter the reaction mixture over filter paper, wash with ethanol, rotary-evaporate to dryness, and take up in a small volume of ethanol, then filter over Fluorosil to remove the last traces of catalyst. Concentrate the resultant oil to dryness with a rotary evaporator (yield 8.64 g, 81% recovery).
3. Dissolve 1.56 g hexahydrofarnesyl acetone in 10 g dry dimethylformamide in a round-bottom flask submerged in ice water and equipped with a dropping funnel containing 1.37 mL triethyl phosphonoacetone. Flush the entire apparatus with argon (Ar). Add triethyl phosphonoacetone over the course of 45 min, followed by 2.2 mL 21% (w/w) sodium ethoxide in ethanol, which is added over the course of 1 h.

4. Remove the mixture from the ice water bath and continue to stir for 48 h under Ar. Analysis by TLC (7:3 hexanes/diethyl ether) should show that the majority of the starting material (R_f 0.5) has reacted (final product R_f 0.6).
5. Transfer the reaction mixture to a separatory funnel with hexane, and wash 2× with a NaCl-saturated solution. Dry the organic layer with $MgS0_4$, pass through filter paper, and evaporate the solvent to dryness by rotary evaporation (yield 1.41 g, 72% recovery).
6. Dissolve 0.75 g of the product from the previous step in 10 mL anhydrous diethyl ether in a round-bottom flask equipped with a stir bar. Cool the apparatus by stirring in an ice-water bath.
7. Add 267 mg of $LiAlH_4$, and stir the reaction mixture overnight under Ar. The following day, add 25 mL saturated NH_4Cl and stir the reaction mixture overnight under Ar.
8. Transfer the reaction mixture to a separatory funnel with diethyl ether and wash with water and saturated NaCl solution. Dry the organic phase with $MgSO_4$, filter through filter paper and concentrate to dryness (yield 440 mg, 51% recovery).
9. Purify the material by TLC (7:3 hexanes–diethyl ether, R_f 0.25), or use in the crude state if proceeding on to make radiolabeled material.

3.1.2. Synthesis of cis-Isomers

1. Form the *cis*-isomers of the allylic alcohols from the respective *trans*-alcohols after oxidation to the aldehyde (*see* **Subheading 3.1.3.**). Allow the mixture of *cis*/*trans* isomers to come to equilibrium (approx 36 *cis*:65 *trans*) by standing at room temperature for several days.
2. Purify the *cis*-isomer by TLC (7:3 hexanes/diethyl ether).

3.1.3. Tritium Labeling of Prenols

1. The method for radiolabeling the prenols is outlined in **Fig. 3**. First, oxidize the alcohol to the aldehyde with an excess of MnO_2. Dissolve 44.7 mg of hexahydrogeranylgeraniol in 2 mL benzene, and add 517 mg of MnO_2. Mix the reaction by tissue culture rotator overnight.
2. The next day, allow the reaction mixture to settle, remove the liquid phase, and filter over glass wool to remove any remaining MnO_2. Add diethyl ether to the original reaction vessel, and repeat the process. Combine the two solutions, and concentrate to dryness under a stream of nitrogen.
3. Chromatograph the material by TLC (7:3 hexanes/diethyl ether, R_f 0.35). Two overlapping bands are present corresponding to the *cis*- and *trans*-double bond isomer, the upper band being the *cis*-isomer and the lower band being the *trans*.
4. Remove the lower one-third of the overlapping bands, elute with diethyl ether, dry with $MgSO_4$, and concentrate to dryness (yield 20%). If not used immediately, this material should be stored at −70°C to prevent isomerization of the *trans*-aldehyde to the equilibrium mixture (~30% *cis*).
5. Elute the remaining material, and rechromatograph if desired.
6. Reduce the aldehyde to the alcohol using $[^3H]NaBH_4$. Dissolve 2 mg of the aldehyde in 1 mL absolute ethanol, and add 5 µL 14 *N* NH_4OH. Dissolve the $[^3H]NaBH_4$

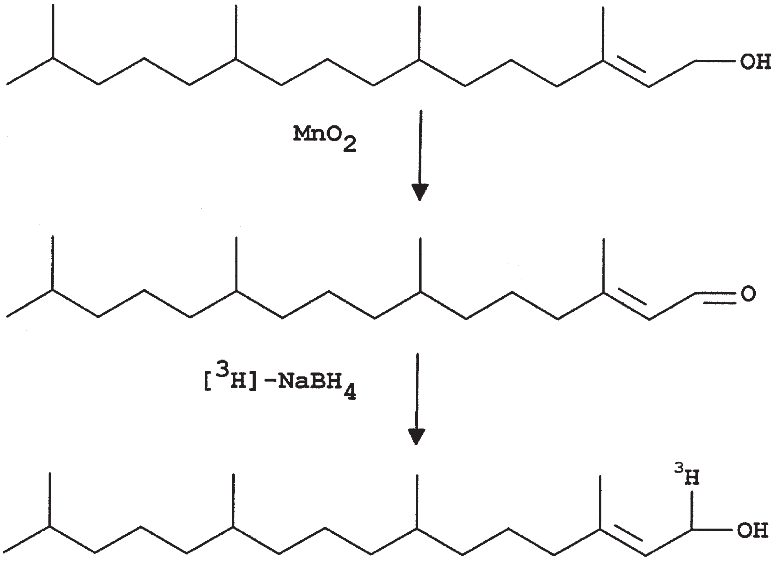

Fig. 3. Schematic for the tritium labeling of naturally occurring prenols.

in ethanol at a concentration of 100 mCi/mL. Add 200 µL of the $[^3H]NaBH_4$ to the aldehyde solution, shake the solution, and then leave it vented in the hood for at least 4 h.

7. Concentrate the material to dryness in a gentle stream of Ar, take up in diethyl ether, and place over a column of silica gel overlaid with $MgSO_4$. Purify the material by TLC (7:3 hexanes/diethyl ether) to remove unreacted starting material and the small amount of the *cis*-isomer that is generated during the reaction. Elute the labeled alcohols from the silica using diethyl ether. Dry using a stream of Ar.
8. Dissolve the product in ethanol, and bring to a final concentration of 2.1 m*M*, 2.6 m*M*, 6.5 m*M*, or 0.4 m*M* for labeled 2 *cis*-GGOH, hexahydrogeranylgeraniol, geraniol, and tetrahydrofarnesol, respectively.

3.2. Cell Culture Experiments

1. Grow HSF and 3T3 cells in monolayers, and maintain in 100-mm Petri dishes at 37°C in a humidified atmosphere of 95% air, 5% CO_2 in DMEM, pH 7.4, supplemented with 10% FCS v/v, 1% (v/v) NEAA, penicillin (100 U/mL), and streptomycin (0.1 mg/mL).
2. Dissociate confluent stock cultures with 0.05% trypsin-0.02% EDTA, and seed HSF cells (3×10^5 cells/35-mm Petri dish) or 3T3 cells (2×10^5 cells/35-mm Petri dish) in a medium containing 0.4% FCS or 1% PDS, respectively, to stop cell replication.
3. HSF cells become quiescent within 3 d and the experiments can begin on d 4. For 3T3 cells, change the medium on d 2 and 4; cells become quiescent within 5 d, and the experiments can begin on d 6.

4. At this time, stimulate the cells by replacing the medium with one containing 10% FCS, in the presence or absence of the tested compounds, and continue the incubation as needed at 37°C. Simvastatin is used at a final concentration of 40 µ*M*, when required.
5. Dissolve unlabeled prenols in absolute ethanol and prepare stock solutions as follows:
   a. 2 m*M* all-*trans*-GGOH (the density for this and all other prenols used in these studies is d^{20} 0.89 g/mL). Add 30 µL of the prenol to 49 mL ethanol and store in 1-mL aliquots at −20°C. On the day of the experiment, dilute an aliquot with ethanol (e.g., 300 µL of GGOH to 900 µL of ethanol) to obtain a working solution of 0.5 m*M*.
   b. 4 m*M* all-*trans*-FOH. Add 20 µL of the prenol to 20 mL of ethanol and store in 1-mL aliquots at −20°C. On the day of the experiment, dilute this 1:3 with ethanol to make 1 m*M* working stock solution.
   c. 4 m*M* geraniol. Add 20 µL of the prenol to 28.83 mL ethanol and store in 1-mL aliquots at −20°C. On the day of the experiment, dilute this 1:3 with ethanol to make 1 m*M* working stock solution.
6. Prenols are light-sensitive, so experiments should be performed under dim light, and incubation should be done with Petri dishes covered with foil. Be careful not to exceed 1% (v/v) ethanol in the culture medium.
7. For estimation of DNA synthesis after mitogenic stimulation, incubate cells for 22–24 h at 37°C then change the medium to one containing 10% FCS and 2 µCi/mL [^{3}H]-thymidine and incubate the cells for another 2 h at 37°C ***(19)***. Remove the medium and wash the monolayers once with PBS at room temperature, then add 2 mL of fresh, ice-cold 5% TCA (w/v), and keep the cells at 4°C on ice for at least 10 min. Remove TCA and wash once with 5% TCA, and dissolve the monolayers in 1 mL of 1 *N* NaOH for 15 min. Transfer 500 µL of the cell lysates to a liquid scintillation vial, add 150 µL glacial acetic acid and 5 mL Aquasol, and measure incorporated radioactivity in a liquid scintillation counter (**Table 1; Fig. 4**).

3.3. Cell Labeling and Prenylated Protein Analysis

3.3.1. Labeling of Proteins with [^{3}H] Farnesol or [^{3}H] Geranylgeraniol and One-Dimensional SDS-PAGE Analysis

1. Dry 9 mCi [^{3}H]-MVA, 500 µCi[^{3}H]-GGOH, or 1 mCi [^{3}H]-FOH, and resuspend in 50 µL ethanol, to bring to the concentration of 4.2 m*M*, 166 µ*M*, or 1.1 m*M*, respectively.
2. Incubate the cells (3 × 35-mm Petri dishes/sample) (*see* **Notes 2–6**) for 20 h at 37°C, with the appropriate labeled isoprenoids. Incubation periods as short as 5 h are sufficient to label most proteins, making it possible to perform time course experiments.
3. To harvest the cells, place the Petri dishes on ice, remove the medium, and wash 3× with ice-cold PBS containing 1 m*M* PMSF. Scrape the cells into 1.5 mL PBS/PMSF per Petri dish, collect the resuspended cells, and centrifuge for 5 min at 200*g*.

Table 1
Mevalonate, Prenols, and Synthetic Analogs Vary in Their Ability to Prevent the Inhibitory Effect of Simvastatin on DNA Synthesisin Cultured Cellsas Measured by Incorporation of [^{3}H]-Thymidine

Cell type	HSF	3T3
Treatment Conditions:	(Reported as % control)	
Simvastatin (S) 40 µ*M*	30	30
S+ mevalonate 100 µ*M*	100	90
S+ all-*trans*-GGOH 5 µ*M*	85	96
S+ 2-*cis*-GGOH 5 µ*M*	29	30
S+ all-*trans*-FOH 10 µ*M*	37	33
S+ geraniol 5 m*M*	29	30
S+ 6,7,10,11,14,15-hexahydro-GGOH 5 µ*M*	34	46
S+ tetrahydro FOH 5 µ*M*	N.A.	36

Experimental conditions: *see* **Subheading 3.2.**
N.A., not assayed.
The mean value of control (100%) without inhibitor was $104 \times 10^3 \pm 3 \times 10^3$ dpm/plate and $187 \times 10^3 \pm 10 \times 10^3$ dpm/plate for HSF and 3T3 cells, respectively.

4. Aspirate the PBS, add 150 µL PBS/PMSF to the cell pellet, and sonicate in a bath sonicator to disrupt the cell pellet. Transfer the resuspended pellet to a 1.5-mL Eppendorf tube.
5. Add 1.3 mL of cold (−20°C) acetone, mix, sonicate, and allow to stand on ice for 15 min. Centrifuge for 5 min (13,000 g at 4°C) to sediment the delipidated proteins. Re-extract the protein pellet twice with 1 mL cold acetone.
6. Add 1 mL chloroform:methanol (2:1), mix, sonicate, and incubate for 30 min at 37°C. Centrifuge for 5 min (13,000 g, 4°C), and collect the lipid extracts.
7. Remove the residual organic solvent by evaporation, and solubilize the delipidated proteins overnight at room temperature in 100 µL 3% SDS, 62.5 m*M* Tris-HCl, pH 6.8.
8. Determine the protein content in 10 µL of sample, according to Lowry ***(27)***.
9. Transfer 10 µL of the sample to a liquid scintillation vial, add 5 mL Aquamix, and measure the incorporated radioactivity.
10. Add 80 µL of sample application buffer to the remainder, and analyze an aliquot (15–80 µg of protein) by one-dimensional SDS-PAGE, according to Laemmli ***(28)***, using a 12% gel.
11. After electrophoresis, wash the gel with destain (methanol–MilliQ water–acetic acid, 25:65:10) for 10 min, then stain the gel (0.1% Coomassie R250 in methanol–MilliQ–acetic acid, 40:50:10) for 20 min, and destain overnight.
12. Treat the gel with Amplify for 30 min in preparation for fluorography. Wash the gel twice with water, dry, and expose to Kodak XOMAT-AR film at −70°C (**Figs. 5** and **6**).

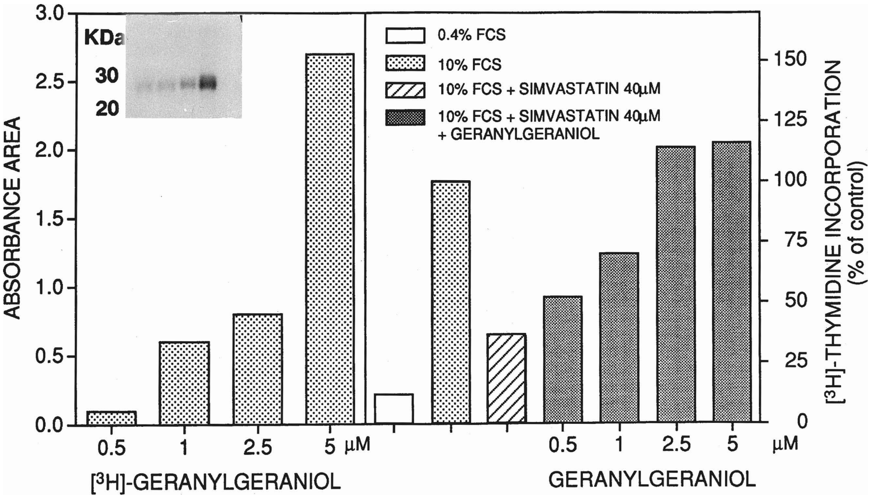

Fig. 4. The ability of simvastatin-treated HSF cells to synthesize DNA was used as a measure of their ability to traverse the cell cycle. HSF cells were seeded at a density of 3 × 10^5/35-mm dish in medium supplemented with 0.4% FCS, and the cultures were incubated for 72 h. Quiescent cells were incubated for 24 h in fresh medium containing 10% FCS, 40 μ*M* simvastatin, and the indicated concentrations of unlabeled GGOH or [^{3}H]-GGOH (15 Ci/mmol). Control cells received only 10% FCS. **(Left panel)** the labeled cell lysates from each treatment group were analyzed by SDS-PAGE (*see* **Subheading 3.3., step 1**), the gel was fluorographed, and the resulting films analyzed by densitometry. The optical density reported for each treatment group represents the sum of all protein bands between 20 and 30 kDa observed in the corresponding gel lane (*see insert*). An equal amount of cell lysate (15 μg cell protein/lane) was applied to each lane. In the experiment shown, 9 × 10^3, 12 × 10^3, 42 × 10^3, and 142 × 10^3 cpm/lane were analyzed for the 0.5, 1, 2.5, and 5 μ*M* [^{3}H]-GGOH samples, for lanes left to right respectively. **(Right panel)** In a parallel experiment, DNA synthesis was determined for each treatment group. Cells were incubated as above, but with a corresponding concentration of unlabeled GGOH. After 22 h, [^{3}H]-thymidine (2 μCi/mL) was added to treated and control samples, and the incubation was continued for another 2 h. Nuclear DNA was analyzed for incorporation of [^{3}H]-thymidine (*see* **Subheading 3.2., step 7**). The incorporation of [^{3}H]-thymidine for each treatment group is reported as a percentage of the control. The mean value of control (100%) was 114 × 10^3 ± 6 × 10^3 dpm/plate.

3.3.2. High-Resolution Two-Dimensional Gel Electrophoresis of Labeled Proteins

1. In 2DE analysis, proteins are first separated on the basis of pI, using isoelectric focusing (IEF), followed by separation on the basis of mol mass. Therefore, differentiation of closely related proteins with similar mol masses is facile ***(29,30)***. Two different IEF methods have been developed for use in high-resolution

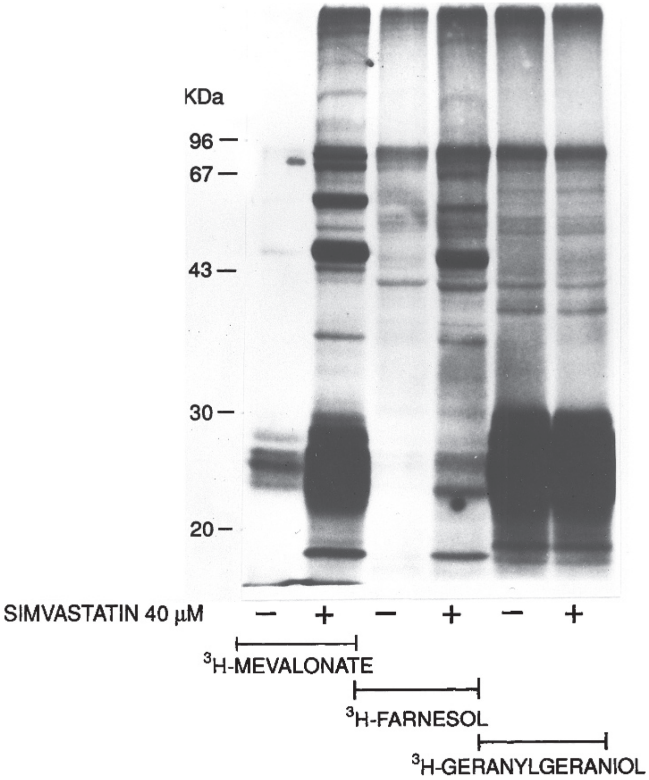

Fig. 5. Metabolic labeling of prenylated proteins in Swiss 3T3 cells after various treatments. Cells were seeded at a density of 2×10^5/dish in medium containing 1% PDS and the cultures incubated for 5 d. Quiescent cells were incubated for 20 h in fresh medium containing 10% FCS, 50 μ*M* [^{3}H]-MVA (35 Ci/mmol), 2.5 μ*M* [^{3}H]-FOH (15 Ci/mmol), or 1 μ*M* [^{3}H] GGOH (60 Ci/mmol), in the presence or absence of 40 μ*M* simvastatin. Cell pellets were delipidated, and equal amounts of cell extracts (80 μg cell protein/lane) were separated by 12% SDS-PAGE and fluorographed. In the experiment shown, 8×10^8, 2.7×10^7, 6×10^7 cpm/lane were analyzed for cells labeled with [^{3}H]-MVA, [^{3}H]-FOH or [^{3}H]-GGOH, respectively.

2DE. The one described here uses carrier ampholytes ***(31–33)*** to obtain the appropriate pH gradient in which sample proteins are focused. This system is available from Genomics Solutions, Chelmsford, MA. The second IEF method uses precast gel strips containing an immobilized pH gradient made by crosslinking ampholytes into a gel matrix ***(29,34)***. This system is available from Amersham Pharmacia, Piscataway, NJ (*see* **Notes 7–10** for additional comments).

2. Prior to analysis by 2DE, cells are labeled as described in **Subheading 3.3.1.**

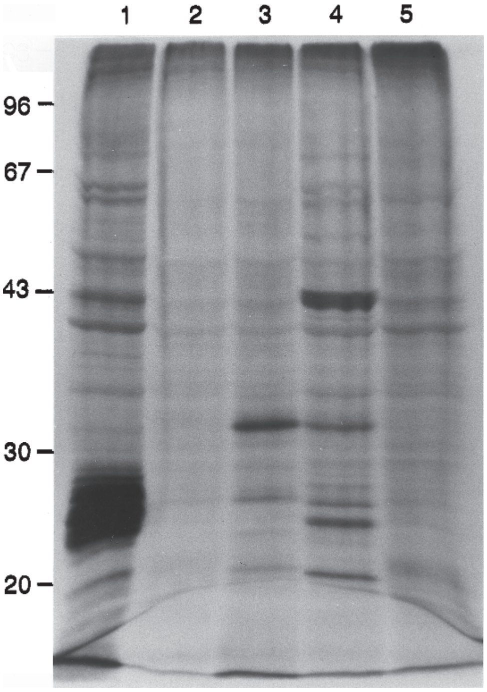

Fig. 6. Metabolic labeling of prenylated proteins in Swiss 3T3 cells after various treatments. Experimental conditions as in **Fig. 5**. Quiescent cells were incubated for 20 h in medium containing 10% FCS, 40 μM simvastatin, and one of the following: lane 1, 5 μM [^{3}H]-all *trans*-GGOH (20 Ci/mmol); lane 2, 5 μM [^{3}H]-*cis, trans*-GGOH (17.5 Ci/mmol); lane 3, 5 μM [^{3}H]-6,7,10,11,14,15 hexahydrogeranylgeraniol (17.5 Ci/mmol); lane 4, 5 μM [^{3}H]-tetrahydrofarnesol (17.5 Ci/mmol); and lane 5, 5 μM [^{3}H]-geraniol (17.5 Ci/mmol). Cell pellets were delipidated, and equal amounts of cell extract (60 µg cell protein/lane) were analyzed by SDS-PAGE on 12% gels, and gels were fluorographed. In the experiment shown, 334×10^3, 197×10^3, 247×10^3, 271×10^3, 285×10^3 cpm/lane were analyzed for lanes 1–5, respectively.

3. On ice, wash each 100-mm dish once with 10 mL PBS, then twice with a solution containing 10 m*M* Tris buffer, pH 7.5, 0.1 m*M* PMSF, and 1.0 µg/mL each of aprotinin, leupeptin, and pepstatin A. Drain for 45 s, and remove residual buffer.
4. Add 240 µL of boiling sample buffer (28 m*M* Tris-HCl, and 22 m*M* Tris base, 0.3% SDS, 200 m*M* DTT, pH 8.0), and collect cells with a cell scraper. Transfer lysate to a 1.5-mL microcentrifuge tube, boil for 5 min, and cool on ice. This and subsequent steps are a modification of methods previously described ***(32,33)***.
5. Add 30 µL (or one-tenth vol) of protein precipitation buffer (24 m*M* Tris base, 476 m*M* Tris-HCl, 50 m*M* $MgCl_2$, 1 mg/mL DNaseI, 0.25 mg/mL RNaseA, pH 8.0) to each cell lysate sample, and incubate on ice for 8 min. Subsequent steps are performed at room temperature, unless otherwise indicated.

6. Add ~6000 cpm of the internal standard, [^{3}H]NEM-labeled soybean trypsin inhibitor (preparation described in **Subheading 3.3.3.**), to each sample (*see* **Note 10**).
7. Add 4 vol of acetone at room temperature to each lysate, and let stand for 20 min. Centrifuge the protein precipitate for 10 min at 16,000 g.
8. Remove the acetone phase, resuspend the precipitate in 800 µL fresh acetone, using a bath sonicator, and centrifuge as above for 5 min. Repeat this wash step once.
9. Let the pellet air dry for 3 min (avoid overdrying), before adding 30 µL of a 1:4 mixture of the SDS buffer (from **Subheading 3.2.2., step 4**) and urea solution (9.9 *M* urea; 4% Triton X-100; 2.2% ampholytes, pH 3–10; 100 m*M* DTT). Warm the sample to 37°C, and bath-sonicate to solubilize the protein (avoid overheating the sample). Centrifuge for 5 min at 16,000 g. Set aside 1 µL for scintillation counting and 1 µL for Bradford protein assay.
10. Apply the sample (26 µL) onto a 1 × 180 mm-tube gel (4.1% T, 0.35% C; ampholyte pH range of 3.0–10.0). Focus for 17.5 h at 1000 V, then for 0.5 h at 2000 V.
11. Extrude the gel from the tube into equilibration buffer (300 m*M* Tris base, 75 m*M* Tris-HCl, 3% SDS, 50 m*M* DTT, and 0.01% bromophenol blue), and incubate it for 2 min before overlaying onto the second dimension SDS-PAGE gel (12.5% T, 0.27% C).
12. Perform SDS-PAGE on large format gels (220 × 240 × 1 mm) for 6 h, using the constant power mode (16 W/gel) in a prechilled tank equipped with Peltier cooling (Genomics Solutions). When five gels are used in this mode, the running buffer temperature will increase 14°C over the course of 6 h when started at 0°C.
13. Fix gels for 1 h in methanol:Milli Q:acetic acid (50:40:10), then stain overnight with a solution containing 0.001% Coomassie R250 in methanol:Milli Q:acetic acid (25:65:10).
14. Immerse each stained gel in 200 mL of Amplify, and incubate for 20 min, with rocking. Without rinsing, dry the treated gel onto heavyweight blotting paper and expose to preflashed Hyperfilm MP for 7–14 d at −70°C. Exposures of >10 wk may be needed to visualize low abundance labeled proteins (**Fig. 7A,B**).

3.3.3. Preparation of the Two-Dimensional Gel Internal Standard, [^{3}H]NEM-Labeled Soybean Trypsin Inhibitor

1. Transfer 100 µL of a freshly prepared solution of 22 m*M* N-ethylmaleimide (NEM) in acetonitrile to a 0.5-mL Reacti-Vial, and add 250 µL [^{3}H]NEM (1 µCi/µL in pentane; 56 Ci/mmol). Mix, and let stand for 2 h uncapped in a fume hood to evaporate the pentane. The final volume will be 110 µL, and the new specific activity will be 95.4 mCi/mmol.
2. Prepare 1 mg/mL solution of soybean trypsin inhibitor (STI) in 50 m*M* Tris, pH 8.5, and reduce the protein by adding DTT (2 m*M*, final concentration). Incubate overnight at 4°C.
3. Remove excess DTT by applying 300 µL of the reduced STI (in six 50-µL aliquots) to six SW rotor equibbed, spin columns, and centrifuge for 4 min at 1000 g in a preequilibrated countertop centrifuge. Prepare spin columns by placing 1 mL

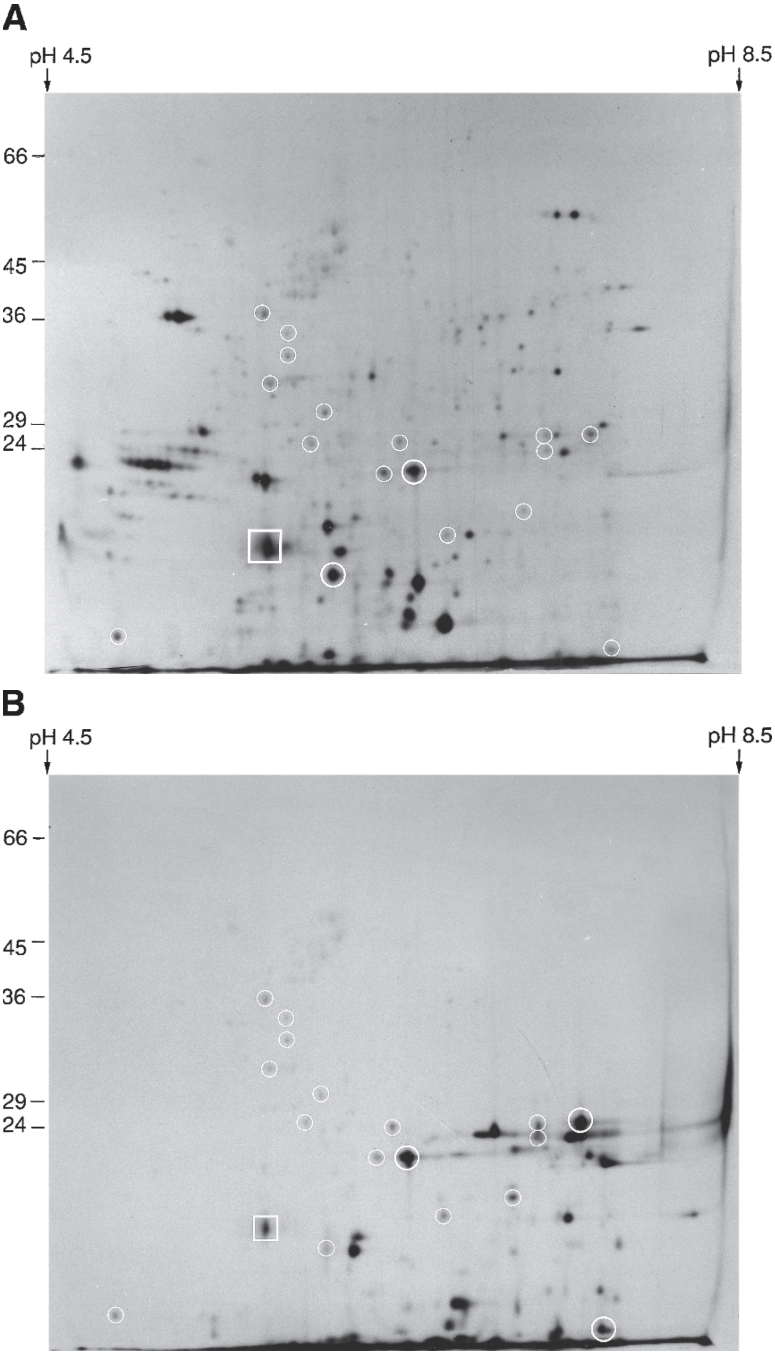
A
pH 4.5
pH 8.5
66
45
36
29
24
B
pH 4.5
pH 8.5
66
45
36
29
24

Fig. 7.

bed-volume of Bio Gel P6-DG (previously rehydrated in 50 m*M* Tris HCl, pH 7.5) into a 1-mL plastic tuberculinsyringe equipped with a plug of glass wool, followed by centrifugation for 2 min at 1000 g. (In addition to removing DTT, this step also permits buffer exchange to provide the proper pH required for the NEM reaction step which follows.)

4. Pool the six column eluates (250–300 µL total volume), and add 10–12 µL of the [^{3}H]NEM solution (24 µCi; *see* **Subheading 3.3.3., step 1**). Incubate for 6 h on ice.
5. Separate the [^{3}H]NEM-labeled STI from the unreacted [^{3}H]NEM using five or six fresh, preequibbelated spin columns (*see* **Subheading 3.3.3., step 3**). Pool the column eluates containing the purified [^{3}H]NEM-labeled STI (1 µCi/nmol protein), divide into 30-µL aliquots, and store at −70°C. To make up a working stock solution, dilute the eluate 1:39 with buffer (6000 cpm/5 µL), and use as an internal standard for 2DE gel fluorography (*see* **Subheading 3.3.2., step 6**; *see also* **Note 10**).

4. Notes

1. Since either all-*trans*-GGOH or MVA, but not all-*trans*-FOH, can completely prevent the inhibitory effect of simvastatin on DNA synthesis in cultured HSF and 3T3 cells (**Table 1**), it may be worthwhile to determine whether this effect is related to the cells' ability to incorporate labeled isoprenoid derivatives into specific proteins, or into other isoprenoid metabolites.
2. To maximize the incorporation of [^{3}H]-MVA into cellular proteins, it is necessary to block endogenous MVA synthesis with a statin such as simvastatin ***(35)***. One-dimensional SDS-PAGE reveals that whole-cell homogenates incorporated [^{3}H]-MVA into at least 10 major bands, with molecular masses ranging from 21 to 72 kDa (**Fig. 5**). Intense bands of radioactivity are seen in the 21–28 kDa range, corresponding to Ras and Ras-related small GTPases ***(2,3)***. In contrast, only a few protein bands are visible when simvastatin is omitted from the incubation.
3. It also is necessary to block endogenous MVA synthesis with simvastatin, for efficient incorporation of [^{3}H]-FOH into specific cellular proteins.

Fig. 7. Two-dimensional gel fluorographs of [^{3}H]-prenol-labeled cell-lysates. HSF cells were labeled with either 5 µm [^{3}H]-FOH or 5 µm [^{3}H]-GGOH (*see* **Subheading 3.3.1.**), lysed, delipidated and analyzed by 2DE and fluorography (*see* **Subheading 3.3.2.**). **(A)** Whole-cell lysate from [^{3}H]-FOH labeled cells that contained 385 µg protein and 170 × 10^3 cpm. Film was exposed for 23 wk **(B)** Whole-cell lysate from [^{3}H]-GGOH-labeled cells that contained 475 µg protein and 390 × 10^3 cpm. Film was exposed for 10 wk. The major proteins that incorporated both FOH and GGOH are circled. At least nine additional clusters of minor proteins display a similar ability to incorporate either prenol, but have not been circled (*see also* **Note 7**). The position of the internal standard, [^{3}H]-NEM-labeled soybean trypsin inhibitor (6250 cpm per gel; *see* **Subheading 3.3.3.**), is indicated by a square.

However, this is not the case for [^{3}H]-GGOH labeling, which proceeds equally well in the presence or absence of simvastatin (**Fig. 5**).

4. In investigations of the cellular uptake of MVA and its derivatives, the authors typically found uptake of less than 0.1% of the added [^{3}H]-MVA, 2% of the added [^{3}H]-FOH, and more than 10–15% of the added [^{3}H]-GGOH. The efficient uptake of GGOH by cells permits rapid labeling of geranylgeranylated proteins. Protein bands from one-dimensional SDS-PAGE are detectable after labeling the cells for only 1–2 h, and subsequent gels require only a few days of film exposure.
5. Both [^{3}H]-GGOH labeling of proteins and the GGOH-mediated prevention of simvastatin-induced inhibition of DNA synthesis show parallel dose-response sensitivities to a similar range of GGOH concentrations (up to 2.5 μ*M*; **Fig. 4**).
6. The labeling approach described here can also be utilized for investigating natural or synthetic isoprenoid analogs (**Fig. 6**). When cells were treated with [^{3}H]-2-*cis*-GGOH (precursor of dolichols), proteins are only weakly labeled, compared to all *trans*-GGOH. Under parallel conditions, the addition of unlabeled 2-*cis*-GGOH failed to prevent the inhibitory effect of simvastatin on DNA synthesis (**Table 1**).
7. When prenylated proteins are selectively labeled with [^{3}H]-FOH or [^{3}H]-GGOH, and analyzed by one-dimensional SDS-PAGE, two subsets of proteins can be identified: a subset of 17 protein bands that incorporate [^{3}H]-FOH, and a separate subset of 12 protein bands that incorporate [^{3}H]-GGOH (*see* **Fig. 5**). However, when similarly labeled proteins are analyzed by 2DE, which allows much greater resolution, a third subset of proteins is identified. Using this approach, 82 proteins are visualized that incorporate only [^{3}H]-FOH, 34 proteins that incorporate only [^{3}H]-GGOH, and 25 proteins that can incorporate either prenol (compare **Fig. 7A,B**), although with varying efficiencies. The presence of unlabeled GGOH in the media during [^{3}H]-FOH labeling experiments or unlabeled FOH during [^{3}H]-GGOH labeling experiments, does not affect the protein composition of these subsets.
8. As mentioned above (*see* **Subheading 3.3.2., step 1**), there are primarily two different methods of IEF available for use in 2DE: one that uses soluble carrier ampholytes ***(31,32)*** and one that uses immobilized ampholytes in Immobiline™ gel-strips, discussed in **Note 9** below ***(29,30,34)***. The analyses presented here were performed using the first method, which was, until recently, the only large format system commercially available. This system is especially convenient for small analytical samples, but can also be used reproducibly for large-sample analyses ***(33)***. When 2DE is coupled with immunoanalysis, this system permits the detection of some proteins that cannot be visualized on blots from Immobiline gels

(M. Aepfelbacker, personal communication; C. Farnsworth, unpublished results).

9. When large samples (>500 µg protein; e.g., cell lysates) are analyzed, the Immobiline system is currently preferable, because of advances in gel technology and sample-handling techniques. These gels are now commercially available in an extended format (0.5 × 3 × 180 mm) and come as dehydrated gel strips bonded to a rigid plastic support. This permits the gel to be rehydrated directly with dilute sample lysates. Additionally, samples containing up to 5 mg protein can be analyzed in this way ***(36)*** without the need for concentration by techniques such as protein precipitation. The gels can accommodate a relatively large sample volume (upper limit of 350 µL vs 26 µL for the carrier ampholyte system). Another advantage of immobilized ampholyte pH gradients is that they allow the use of very narrow pH gradients (e.g., pH 5.5–6.5). This permits the separation of charge-isoforms that differ by only pH 0.05 units ***(29)***. Lastly, when 2DE is coupled with immunoanalysis and overlay assays, detailed maps of multimember protein families can be constructed ***(37)***.
10. In control experiments using lysates of unlabeled cells, recovery of the labeled internal standard, [^{3}H]-NEM STI (*see* **Subheading 3.3.3.**), following complete sample workup (*see* **Subheading 3.3.2., steps 6–9**) was 91 ± 4%, *n* = 3.

Acknowledgment

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The Metabolic Labeling and Analysis of Isoprenylated Proteins

Douglas A. Andres, Dean C. Crick, H. Peter Spielmann, and Charles J. Waechter

1. Introduction

1.1. Background

The posttranslational modification of proteins by the covalent attachment of farnesyl and geranylgeranyl groups to cysteine residues at or near the carboxyl-(C)-terminus via a thioether bond is now well established in mammalian cells ***(1–6)***. Most isoprenylated proteins are thought to serve as regulators of cell signaling and membrane trafficking. Farnesylation and geranylgeranylation of the cysteinyl residues has been shown to promote both protein–protein and protein–membrane interactions ***(3,7,8)***. Isoprenylation, and in some cases the subsequent palmitoylation, provide a mechanism for the membrane association of polypeptides that lack a transmembrane domain, and appear to be prerequisite for their in vivo activity ***(3,9,10)***.

Three distinct protein prenyltransferases catalyzing these modifications have been identified ***(1,2,4–6)***. Two geranylgeranyltransferases (GGTases) have been characterized and are known to modify distinct protein substrates. The CaaX GGTase (also known as GGTase-1) geranylgeranylates proteins that end in a CaaL(F) sequence, where C is cysteine, A is usually an aliphatic amino acid, and the C-terminal amino acyl group is leucine (L) or phenylalanine (F). Rab GGTase (also known as GGTase-2) catalyzes the attachment of two geranylgeranyl groups to paired C-terminal cysteines in most members of the Rab family of GTP-binding proteins ***(11)***. These proteins terminate in a Cys-Cys, Cys-X-Cys, or Cys-Cys-X-X motif where X is a small hydrophobic amino acid. Another set of regulatory proteins is modified by protein farnesyltransferase (FTase). All known farnesylated proteins terminate in a tetrapeptide CaaX box, wherein

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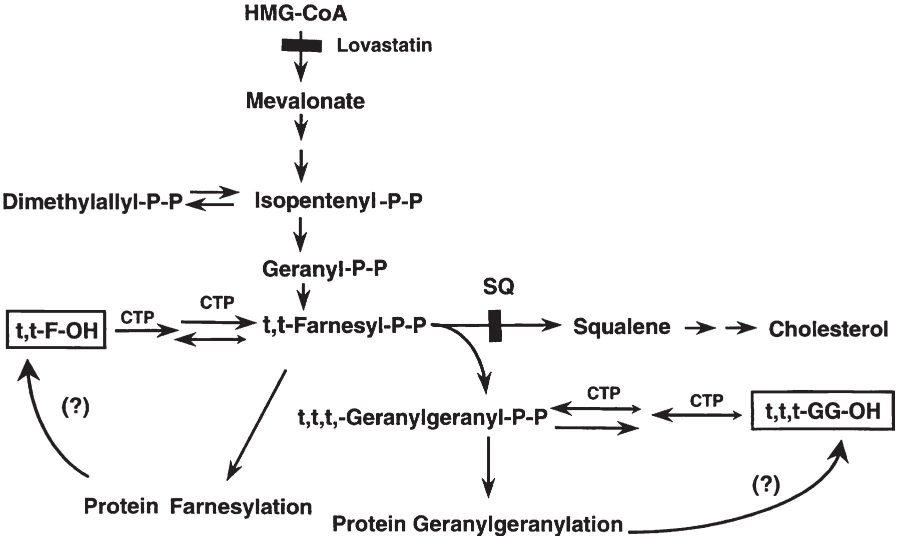
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Lovastatin
Mevalonate
Dimethylallyl-P-P
Isopentenyl -P-P
Geranyl-P-P
SQ
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CTP
CTP
t,t-Farnesyl-P-P
Squalene
Cholesterol
(?)
t,t,t,-Geranylgeranyl-P-P
CTP
CTP
t,t,t-GG-OH
(?)
Protein Farnesylation
Protein Geranylgeranylation

Fig. 1. Proposed "salvage" pathway for the utilization of F-OH and GG-OH for isoprenoid biosynthesis. Evidence for the presence of microsomal enzymes catalyzing the conversion of F-P-P and GG-P-P to the free isoprenols has been reported by Bansal and Vaidya *(13)*.

C is cysteine, A is an aliphatic amino acid, and X has been shown to be a COOH-terminal methionine, serine, glutamine, cysteine, or alanine.

Isoprenylated proteins are commonly studied by metabolic labeling of cultured cells by incubation with [^{3}H]mevalonate which is enzymatically converted to [^{3}H]farnesyl pyrophosphate (F-P-P) and [^{3}H]geranylgeranyl pyrophosphate (GG-P-P) prior to being incorporated into protein (***ref. 12*; Fig. 1**). Cellular proteins can then be analyzed by gel electrophoresis and autoradiography. However, [^{3}H]mevalonate labeling does not distinguish between farnesylated and geranylgeranylated proteins directly. Other methods for the identification of the isoprenyl group attached to protein that make use of [^{3}H]mevalonolactone labeling have been described. These methods generally require the isolation of large amounts of labeled protein, extensive proteolysis, a number of column chromatographic steps, and cleavage of the thioether bond with Raney nickel or methyl iodide, followed by gas chromatography or HPLC and mass spectrometry to identify the volatile cleavage products (*see* ***ref. 4*** and papers cited therein).

This chapter describes the use of either the natural free [^{3}H]farnesol (F-OH) and [^{3}H]geranylgeraniol (GG-OH) or the unnatural and non-radioactive anilinogeraniol (AGOH) in the selective metabolic labeling of farnesylated and geranylgeranylated proteins in cultured mammalian cells. In addition, the methods use materials and equipment readily available in most laboratories.

We have recently shown that mammalian cells can utilize the free isoprenols, GG-OH and F-OH, for the isoprenylation of cellular proteins ***(14,15)***. When C6 glioma cells were incubated with [^{3}H]F-OH, radioactivity was also incorporated into cholesterol ***(14)***. The observation that the incorporation of label into sterol was blocked by squalestatin 1 (SQ), a potent inhibitor of squalene synthetase ***(16–20)***, suggested that F-OH, and probably GG-OH, are utilized for isoprenoid biosynthesis after being converted to the corresponding activated allylic pyrophosphates, F-P-P and GG-P-P (**Fig. 1**). Preliminary studies have suggested the presence of enzyme systems in mammals and lower organisms that are capable of phosphorylating F-OH and GG-OH ***(21–23)***. More recently microsomal fractions from *N. tabacum* have been shown to contain CTP-mediated kinases that catalyze the conversion of F-OH and GG-OH to the respective allylic pyrophosphate intermediates by two successive monophosphorylation reactions (24). Further work is certainly warranted on the isolation and characterization of these enzymes. The early developments in understanding the mechanism and physiological significance of the salvage pathway for the utilization of F-OH and GG-OH have been reviewed ***(25)***.

A drawback to all metabolic labeling approaches to examine protein isoprenylation is the inherently low sensitivity of autoradiographic detection of [^{3}H]-labeled proteins and the limitation that incorporation of radiolabeled prenyl groups does not provide a convenient method for the isolation of the modified proteins. To address these limitations, incorporation of unnatural F-P-P analogues has been utilized to examine the prenylation status of cellular proteins. These methods provide a facile approach to both the detection and isolation of modified proteins ***(26–28)***. These methods include a tagging-via-substrate (TAS) approach using *in vivo* incorporation of 8-azido-farnesol ***(27)*** in conjunction with a biotinylated phosphine capture reagent to isolate modified proteins, which are then identified by mass spectroscopy. However, the TAS technology requires access to specialized equipment and thus does not lend itself to the routine detection of prenylated proteins. Here, we describe a more convenient method to simplify the detection of unnatural F-P-P modified proteins, making use of analogue-selective antibodies. In particular, recent studies have shown that the unnatural F-P-P analogue anilinogeranyl diphosphate (AG-P-P) is transferred by FTase to protein substrates with kinetics similar to F-P-P, that AG-OH is effectively utilized in a wide range of cells for the *in vivo* labeling of farnesylated proteins, and does not resemble any known natural protein modification ***(27,29,30)***. More importantly, anilinogeranyl modified proteins can be readily detected with anti-anilinogeranyl antibodies ***(28)***. Thus, the unnatural FPP analog and corresponding antibodies provide a simple and rapid method for monitoring FTase activity in cells and detection of cellular proteins modified by AG-OH.

1.2. Experimental Strategy

Utilizing free F-OH or GG-OH as the isotopic precursors of F-P-P and GG-P-P, or AG-OH as an unnatural alternative FTase substrate, has several experimental advantages over metabolic labeling of isoprenylated proteins with mevalonate. First, isoprenoid alcohols are more hydrophobic and rapidly enter cultured mammalian cells. They are efficiently utilized in a range of mammalian cell lines, and obviate the need to include HMG-CoA reductase inhibitors to lower endogenous pools of mevalonate. The experimental strategy is illustrated in **Fig. 1**. A key advantage of the strategy is that F-OH, AGOH and GG-OH are selectively incorporated into distinct subsets of isoprenylated proteins, providing a simple and convenient approach to specifically label farnesylated or geranylgeranylated proteins (**Figs. 2** and **8**).

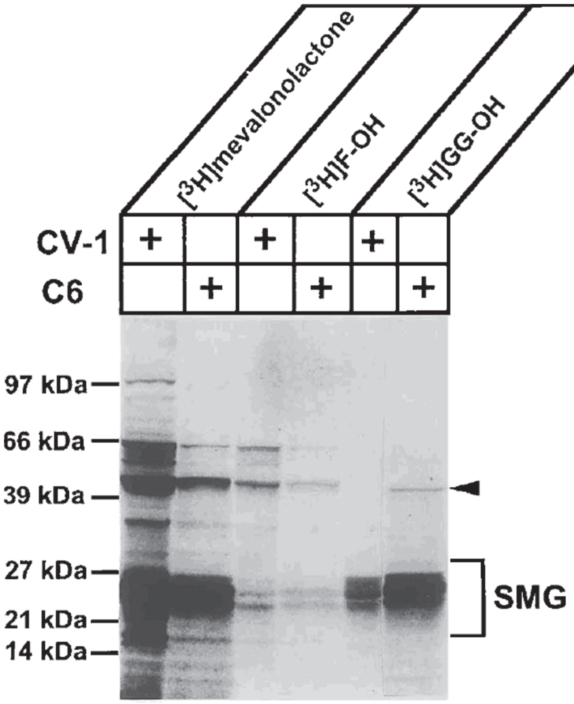

Fig. 2. SDS-PAGE analysis of proteins labeled by incubating C6 glioma cells and CV-1 cells with [^{3}H]mevalonate, [^{3}H]F-OH, or [^{3}H]GG-OH. The details of the metabolic labeling procedure and SDS-PAGE analysis are described in **Subheadings 3.1.–3.3.** For these analyses proteins were metabolically labeled by incubating the indicated cultured cells with [^{3}H]F-OH in the presence of lovastatin (5 µg/mL) because it increased the amount of radioactivity incorporated into protein during long-term incubations, presumably by reducing the size of the endogenous pool of F-P-P. The gel patterns reveal that distinctly different sets of proteins are labeled by each precursor in C6 and CV-1 cells. Consistent with selective labeling by [^{3}H]F-OH and [^{3}H]GG-OH, the labeling pattern for [^{3}H]mevalonate, which can be converted to ^{3}H-labeled F-P-P and GG-P-P, appears to be a composite of the patterns seen with the individual [^{3}H]isoprenols. SMG = proteins in the size range (19–27 kDa) of small GTP-binding proteins. (Figure reprinted with permission from ***ref. 14***).

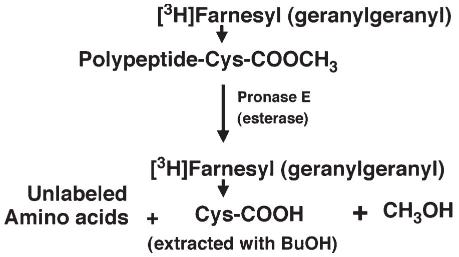

Fig. 3. Experimental scheme for the rapid analysis of metabolically labeled isoprenylated cysteine residues labeled by various isotopic precursors. The experimental details of this procedure are described in **Subheading 3.4.**

Following metabolic labeling using [^{3}H]F-OH, [^{3}H]GG-OH, or [^{3}H]mevalonolactone, the metabolically labeled proteins are exhaustively digested with Pronase E to liberate the specific isoprenyl-cysteine residues (**Fig. 3**). The identity of the isoprenylated cysteine residue can then be readily identified by normal or reverse-phase thin layer chromatography (TLC). **Figs. 4** and **5** show representative TLC analysis of isoprenyl-cysteines released from metabolically labeled cellular proteins and from recombinant proteins that were isoprenylated in vitro. Alternatively, once the non-radioactive AG-OH is incorporated in cellular proteins, total cell lysates can be directly subjected to immunoblotting with anti-AG antibodies to detect AG-modified endogenous proteins (**Fig. 8**). The anilinogeranyl moiety is structurally unrelated to any other known cellular components or protein modifications making it an excellent epitope for selective antibody recognition (Troutman Bioconj Chem 2005REF). The AG-P-P prodrug anilinogeraniol (AG-OH) is incorporated into cellular protein in the presence of the endogenous F-P-P pool without the requirement for blocking endogenous FPP formation and is remarkably non-toxic *(28)*. Thus, AG-OH and the corresponding antibodies provide a simple, rapid, and nonradioactive method for monitoring FTase activity and detection of cellular proteins modified by AG-OH.

The isoprenol labeling and Pronase E methods may also be applied to the analysis of individual metabolically labeled proteins. These methods provide a simple and convenient approach for the identification of the isoprenyl group found on a specific protein. Two experimental approaches are available. In the first, separate cell cultures are incubated with [^{3}H]mevalonate, [^{3}H]F-OH, or [^{3}H]GG-OH the metabolically labeled protein of interest is then purified and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The specificity of the F-OH and GG-OH incorporation allows the identity of the prenyl group to be directly assessed (**Fig. 6**). In the second, the cells are metabolically labeled with [^{3}H]mevalonolactone and the isolated protein of interest is subjected to Pronase E treatment followed

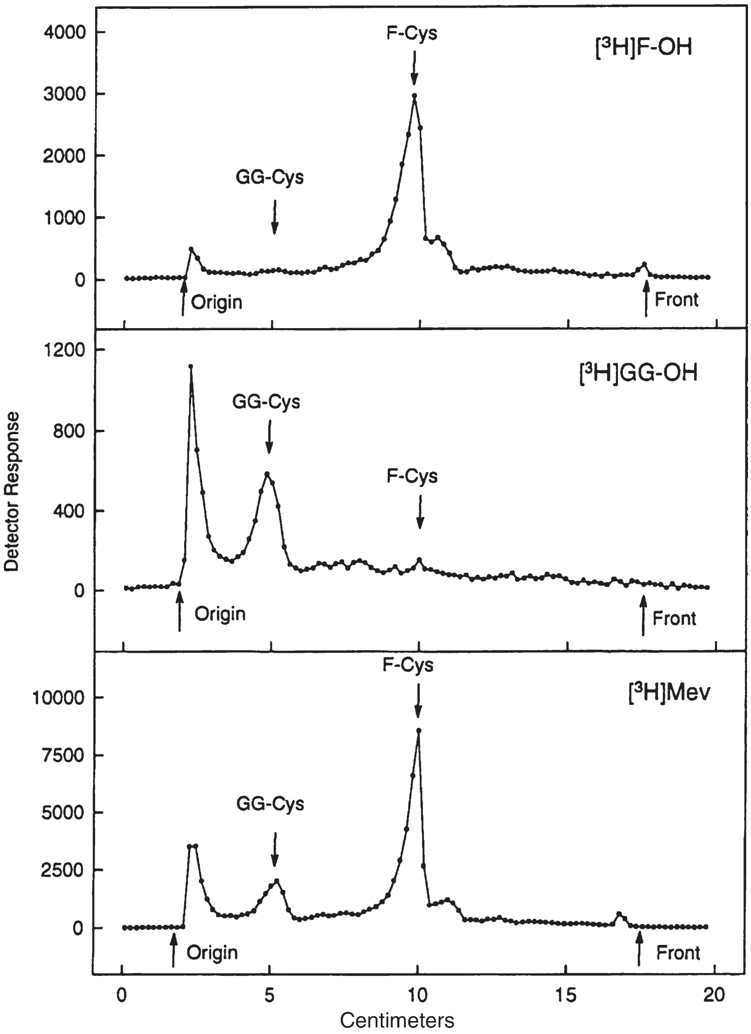

Fig. 4. Chromatographic analysis of isoprenyl-cysteine residues metabolically labeled by incubating C6 glioma cells with, [^{3}H]F-OH (*upper panel*), [^{3}H]GG-OH (*middle panel*) or [^{3}H]mevalonate (*lower panel*). From the traces illustrated, it can be seen that F-Cys and GG-Cys are both metabolically labeled when C6 cells are incubated with [^{3}H]mevalonate, but only F-Cys or GG-Cys are labeled when C6 cells are incubated with [^{3}H]F-OH or [^{3}H]GG-OH, respectively. Virtually identical results were obtained by chromatographic analysis of the Pronase E digests of CV-1 proteins metabolically labeled by each isotopic precursor. Radiolabeled products are also observed at the origin of the TLC which could be incompletely digested isoprenylated peptides, or in the case of [^{3}H]mevalonate and [^{3}H]GG-OH, possibly mono- or digeranylgeranylated Cys-Cys or Cys-X-Cys sequences reprinted with permission *(14)* (*see* **Fig. 5**).

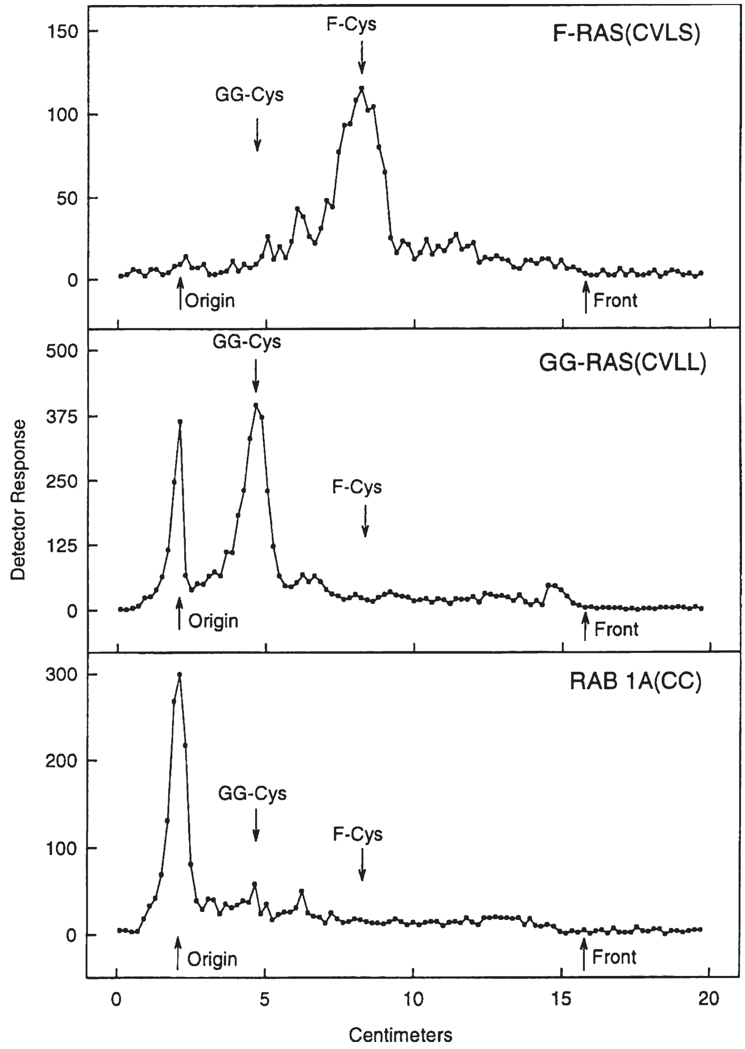

Fig. 5. Chromatographic analyses of isoprenyl-cysteine residues liberated by Pronase E digestion of recombinant protein substrates enzymatically labeled in vitro by [^{3}H]F-P-P or [^{3}H]GG-P-P. The recombinant proteins were labeled by incubation with recombinant FTase (*upper panel*), GGTase I (*upper panel*) or GGTase II (*lower panel*), essentially under the conditions described previously *(18)*.

by TLC analysis of the butanol-soluble products. In this case analysis the chromatogram reveals the nature of the isoprenyl group (**Fig. 7**). The experimental methods for these analytical procedures are presented in detail in this chapter.

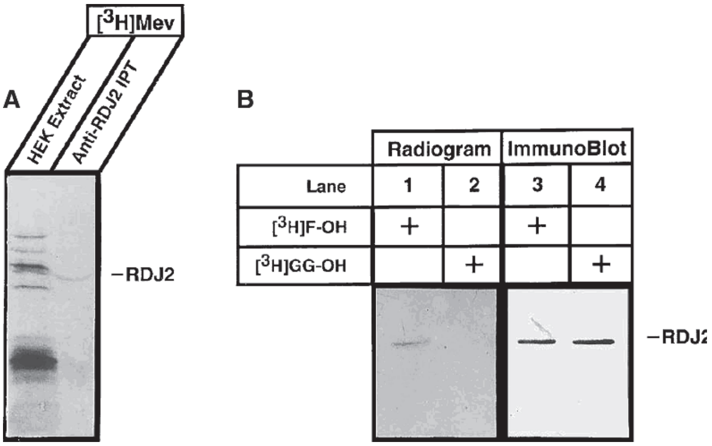

Fig. 6. Specific metabolic labeling of RDJ2-transfected HEK cells. **(A)** Monolayers of human embryonic kidney HEK cells transfected with pRDJ2 (an expression plasmid that contained the RDJ2 cDNA under the control of a CMV promoter) were radiolabeled with [^{3}H]mevalonate and the expressed RDJ2 immunoprecipitated from detergent-solubilized cell extracts using a RDJ2-specific antibody. A portion of the resulting immunoprecipitate as well as a portion of the cell extract were subjected to SDS-PAGE. The gel was treated with Amplify, dried, and exposed to film for 14 d. **(B)** Immunoprecipitated RDJ2 after metabolic labeling of transfected HEK cells with [^{3}H]F-OH (*lane 1*) or [^{3}H]GG-OH (*lane 2*) was subjected to SDS-PAGE analysis and fluorography. The remaining immunoprecipitated protein fractions isolated from HEK cells after metabolic labeling with [^{3}H]F-OH (*lane 3*) or [^{3}H]GG-OH (*lane 4*) were immunoblotted using anti-RDJ2 IgG and subjected to chemiluminescence detection.

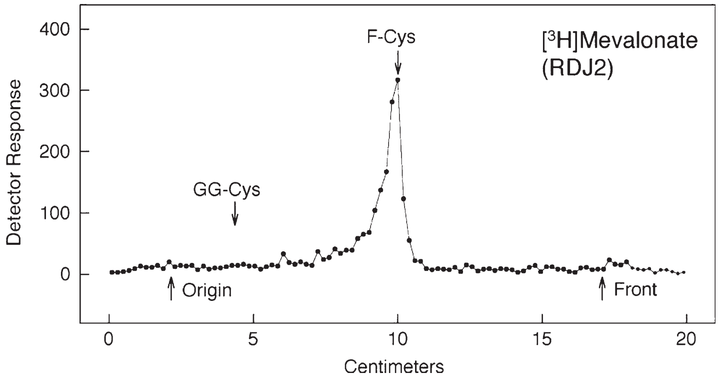

Fig. 7. Chromatographic analysis of butanol-soluble products released by Pronase E digestion of RDJ2 protein metabolically labeled by incubation with [^{3}H]mevalonolactone. Immunoprecipitated RDJ2, isolated from [^{3}H]mevalonolactone-labeled HEK cells, was subjected to Pronase E digestion. The labeled products were extracted with 1-butanol, and analyzed by reverse-phase chromatography using C18 reverse-phase TLC plates, and developed in acetonitrile–water–acetic acid (75:25:1). Radioactive zones were located with a Bioscan Imaging System 200-IBM. The *arrows* indicate the position of authentic F-Cys and GG-Cys.

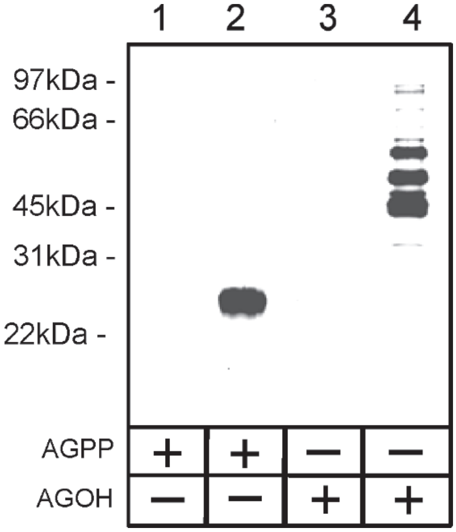

Fig. 8. Incorporation and detection of anilinogeranyl modified proteins by immunoblotting. *In vitro* prenylation reactions were performed using recombinant farnesyltransferase and AGPP in the absence (*lane 1*) or presence (*lane 2*) of H-Ras. The reactions were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting with anti-AG polyclonal antibody ***(28, 33)***. *In vivo* prenylation reactions (*lanes 3* and *4*) were performed on HEK-293 cells preincubated with 30 μ*M* lovostatin and 10 μ*M* GGOH for 24 h. The media was removed and replenished with media containing either 3 μ*M* FTI-277 (*lane 3*) or no inhibitor (*lane 4*) for 1 h. AGOH (100 μ*M*) was then added directly to the cultures and cells incubated for an additional 2 h. Cells were harvested and total cell lysates (50 μg) resolved by SDS-PAGE and anilinogeranyl modified endogenous proteins detected by immunoblotting using anti-AG polyclonal antibody.

2. Materials

2.1. Metabolic Radiolabeling of Mammalian Cells in Culture with [^{3}H]Farnesol, [^{3}H]Geranylgeraniol, and [^{3}H]Mevalonolactone

1. ω,*t*,*t*-[1-^{3}H]Farnesol (20 Ci/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO).
2. ω,*t*,*t*,*t*-[^{3}H]Geranylgeraniol (60 Ci/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO).
3. [^{3}H]Mevalonolactone (60 Ci/mmol, American Radiolabeled Chemicals, Inc., St. Louis, MO).
4. Appropriate cell culture media, plastic ware, and cell incubator.
5. 95% Ethanol.
6. Serum Supreme (SS), an inexpensive fetal bovine serum (FBS) substitute obtained from BioWhittaker, has been successfully used with C6 glioma, Chinese hamster ovary (CHO) clone UT-2 and green monkey kidney (CV-1) cells in this laboratory.
7. Bath sonicator.

2.2. Delipidation of Labeled Proteins

1. Phosphate-buffered saline (PBS).
2. PBS containing 2 m*M* EDTA.
3. Methanol.
4. Chloroform–methanol (2:1, v/v).
5. 12-mL Disposable conical screw-capped glass centrifuge tubes.
6. Benchtop centrifuge.
7. Probe sonicator.

2.3. SDS-PAGE Analysis of Metabolically Labeled Proteins

1. SDS-PAGE apparatus.
2. Western blot transfer apparatus.
3. Nitrocellulose membrane (Schleicher and Schuell, Protran BA83).
4. 2% SDS, 5 m*M* 2-mercaptoethanol.
5. Ponceau S: 0.2% Ponceau S, 3% trichloracetic acid (TCA), 3% sulfosalicylic acid (Sigma, S 3147).
6. Fluorographic reagent (Amplify, Amersham Corp.).
7. Sheets of stiff plastic (such as previously exposed X-ray film).

2.4. Pronase E Digestion and Chromatographic Analysis of Radiolabeled Proteins

1. *N*-[2-hydroxyethyl]piperazine-*N*′-[2′ -ethanesulfonic acid] Hepes, pH 7.4.
2. Calcium acetate.
3. Bath sonicator.
4. Pronase E (Sigma, St. Louis, MO).
5. 37°C water bath.
6. *n*-Butanol saturated with water.
7. Bench centrifuge.
8. Oxygen-free nitrogen gas.
9. Chloroform:methanol:H_2O (10:10:3, by vol).
10. Farnesyl-cysteine (F-Cys) was synthesized as described by Kamiya et al. ***(31)***.
11. Geranylgeranyl-cysteine (GG-Cys) was synthesized as described by Kamiya et al. ***(31)*** except that isopropanol is substituted for methanol in the reaction solvent to improve the yield of the synthetic reaction.
12. Silica Gel G 60 TLC plates (Sigma, St. Louis, MO).
13. Chloroform:methanol:7 *N* ammonia hydroxide (45:50:5).
14. Si*C_{18} reverse-phase plates (J. T. Baker Inc., Phillipsburg, NJ).
15. Acetonitrile:H_2O:acetic acid (75:25:1, by vol).
16. Conical glass tubes.
17. Anisaldehyde spray reagent (32) (*see* **Note 1**).
18. Ninhydrin spray reagent (*see* **Note 2**).

2.5. Immunoprecipitation of Specific Radiolabeled Protein

1. Protein-specific immunoprecipitating antibody.
2. Protein A-Sepharose (Pharmacia).

3. Wash buffer A: 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.2% Nonidet P-40 (NP-40).
4. Wash buffer B: 20 m*M* Tris-HCl, pH 7.5, 500 m*M* NaCl, 0.2% NP-40.
5. Wash buffer C: 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
6. *n*-Butanol saturated with water.
7. Tabletop ultracentrifuge (Beckman).

2.6. Immunodetection of AG-Modified Cellular Proteins

1. Anilinogeraniol (30 µ*M*, AG-OH) (Contact Drs. H. P. Spielmann and D. A. Andres, Univ. Kentucky, Lexington, KY) ***(28,33,34)***.
2. Appropriate cell culture media, plastic ware, and cell incubator.
3. Cell lysis buffer: 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40), 1 m*M* phenylmethyl+sulfonyl fluoride (PMSF) (add fresh PMSF solution and lysis is carried out at 4°C). Bradford reagent protein concentration kit (Pierce).
4. AG-specific antibody (Contact Drs. H. P. Spielmann and D. A. Andres, Univ. Kentucky, Lexington, KY) ***(28,33)***.
5. SDS-PAGE apparatus.
6. Western blot transfer apparatus.
7. Nitrocellulose membrane (Schleicher and Schuell, Protran BA83).
8. Blocking buffer PCT: 1 × phosphate buffered saline (PBS), 0.1 % Tween 20, 1% casein
9. Shaker platform.
10. Wash buffer PT: 1× PBS and 0.1% Tween 20.
11. Wash buffer PTE: 1× PBS and 0.3% Tween 20.
12. horseradish peroxidase-conjugated secondary antibody (Zymed).
13. Enhanced chemiluminescent detection reagent (Pierce).

3. Methods

3.1. Procedure for the Selective Metabolic Labeling of Farnesylated and Geranylgeranylated Proteins

Procedures are described for the metabolic labeling of mammalian cells grown to near-confluence in Falcon 3001 tissue culture dishes. These protocols can be scaled up or down as appropriate. The incorporation of [^{3}H]F-OH or [^{3}H]GG-OH into protein was linear with respect to time and the concentration of [^{3}H]isoprenol in tissue culture dishes ranging from 10 to 35 mm in diameter under the conditions described here ***(14,15,25)*** (*see* **Note 3**).

1. For metabolic labeling experiments with either [^{3}H]F-OH or [^{3}H]GG-OH, a disposable conical glass centrifuge tube (screw capped) and Teflon-lined cap are flame sterilized. The labeled isoprenol dissolved in ethanol is added to the tube and the ethanol is evaporated under a sterile stream of air.
2. An appropriate volume of sterile SS is added to yield a final concentration of 0.5–1 mCi/mL. The labeled isoprenols are dispersed in the SS by sonication in a Branson bath sonicator for 10 min. After sonication an aliquot is taken for liquid scintillation counting to verify that the ^{3}H-labeled isoprenol has been quantitatively dispersed in SS.

3. The growth medium is removed from the cultured cells by aspiration and 500 µL of labeling medium, consisting of an appropriate medium and SS (final concentration = 3–5%) containing [^{3}H]GG-OH or [^{3}H]F-OH, is added.
4. Cell cultures are typically incubated at 37°C under 5% CO_2 for 6–24 h. Actual culture media and incubation conditions will vary depending on the specific cell type being studied.

3.2. Recovery of Metabolically Labeled Proteins from Adherent Cell Lines

1. The labeling medium is removed by gentle aspiration.
2. The metabolically labeled cells are washed with 1–2 mL of ice-cold PBS, to remove unincorporated isotopic precursor.
3. One milliliter of PBS containing 2 m*M* EDTA is added, and cells are incubated for 5 min at room temperature. The washed cells are gently scraped from the dish with a disposable cell scraper, and transferred to a 12-mL conical glass centrifuge tube.
4. The metabolically labeled cells are sedimented by centrifugation (500*g*, 5 min), and the PBS–EDTA is removed by aspiration (avoid disrupting the cell pellet).
5. The cells are resuspended in PBS (1–2 mL) and the PBS is removed by aspiration after the cells are sedimented by centrifugation.
6. Two milliliters of methanol (CH_3OH) is added to the cell pellet, and the pellet is disrupted by sonication using a probe sonicator.
7. The suspension is sedimented by centrifugation (1500 *g* for 5 min).
8. The CH_3OH extract is carefully removed, to avoid disturbing the partially delipidated pellet, and transferred to a glass conical tube (*see* **Note 4**).
9. The protein pellet is reextraced twice with 2 mL of $CHCl_3$–CH_3OH (2:1), and the extracts are pooled with the CH_3OH extract (*see* **Note 4**).
10. Residual organic solvent is removed from the delipidated protein pellet by evaporation under a stream of nitrogen. The delipidated protein fractions are then subjected to Pronase E digestion for analysis of isoprenyl-cysteine analysis by TLC (*see* **Subheading 3.4**), or dissolved in 2% SDS, 5 m*M* 2-mercaptoethanol for SDS-PAGE analysis.

3.3. SDS-PAGE Analysis of Metabolically Labeled Proteins

To examine the molecular weight and number of proteins metabolically labeled by incubation with [^{3}H]F-OH or [^{3}H]GG-OH, the delipidated protein fractions can be analyzed by SDS-PAGE. Because these experiments rely on the detection of low-energy ^{3}H-labeled compounds, two procedures are described for the use of fluorography to increase the sensitivity of detection (*see* **Note 5** before proceeding).

1. The delipidated protein fractions are solubilized in 2% SDS, 5 m*M* β-mercaptoethanol. An aliquot is used to determine the amount of labeled precursor incorporated into protein.
2. The radiolabeled polypeptides (20–60 µg of protein) were analyzed by SDS-PAGE using an appropriate percentage polyacrylamide resolving gel (4–20%) for the proteins of interest.

3. Following SDS-PAGE, gels can be analyzed using two distinct methods. In the first, the gel is directly soaked in the fluorographic reagent, Amplify (Amersham), according to the manufacturer's protocol, dried, and exposed to X-ray film as described in **step 5**. In a second approach, proteins were electrophoretically transferred to nitrocellulose filters and stained with Ponceau S to determine the efficiency of transfer. The nitrocellulose filters are destained by brief washing in distilled water and allowed to air-dry. (*See* **Note 5** for a discussion of the merits of each method before continuing.)
4. The filters were then dipped briefly in the fluorographic reagent, Amplify (Amersham), placed on a sheet of plastic backing, and dried for 1 h at 50°C. It is important that a thin and even film of Amplify reagent remain on the filter and that it be placed protein side up to dry.
5. Fluorograms were produced by exposing preflashed X-ray film to the nitrocellulose filter, or dried SDS-PAGE gel, for 5–30 d at −80°C.

3.4. Methods for the Identification of Cysteine-Linked Isoprenyl Group

These simple methods are inexpensive, rapid, and allow the identification of the isoprenyl-cysteine residue(s) from isoprenylated protein(s). Examples of this method for the identification of isoprenyl-cysteine groups from metabolically labeled cells and recombinant proteins labeled in vitro are shown in **Figs. 4** and **5**. As expected, [^{3}H]F-Cys and [^{3}H]GG-Cys were liberated from RAS(CVLS) and RAS(CVLL), respectively. A radioactive peak is also seen at the origin in the analysis of the Pronase digest of radiolabeled RAS(CVLL) (**Fig. 5**, *middle panel*). This radiolabeled product(s) is probably incompletely digested [^{3}H]geranylgeranylated peptides. Rab 1A terminates in two cysteine residues, both of which are isoprenylated (35). **Fig. 5** (*lower panel*) indicates that Pronase E is incapable of cleaving between the two cysteine residues.

1. To liberate the labeled isoprenyl-cysteine residues for analysis, the delipidated protein fractions (50–100 µg) are incubated with 2 mg of Pronase E; 50 m*M* HEPES, pH 7.4; and 2 m*M* calcium acetate in a total volume of 0.1 mL at 37°C for approx 16 h. The experimental scheme for this analysis is illustrated in **Fig. 3** (*see* **Note 6**).
2. Proteolysis is terminated by the addition of 1 mL of *n*-butanol saturated with H_2O and mixing vigorously.
3. Centrifuge the mixture for 5 min in a benchtop centrifuge at 1500*g*. Two phases will form, and the upper phase should be clear (*see* **Note 7**).
4. Transfer the upper phase, containing *n*-butanol, to a separate conical glass centrifuge tube.
5. Add 1 mL of H_2O to the butanol extract and mix vigorously. Centrifuge at 1500*g* for 5 min, to effect a phase separation. Remove the lower aqueous phase with a Pasteur pipet (*see* **Note 8**). Evaporate the *n*-butanol under a stream of nitrogen at 30–40°C (*see* **Note 9**).

6. The labeled isoprenyl-cysteines are dissolved in 250 µL of $CHCl_3$:CH_3OH:H_2O (10:10:3, by vol) by mixing vigorously, and an aliquot (10 µL) is taken to determine the amount of radioactivity.
7. The radiolabeled products are analyzed chromatographically on a normal-phase system, using silica gel G 60 TLC plates developed in $CHCl_3$:CH_3OH:7 *N* NH_4OH (45:50:5, by vol) or by reverse-phase chromatography using silica gel Si*C18 reverse-phase plates developed with acetonitrile–H_2O–acetic acid (75:25:1, by vol) (*see* **Note 10**).
8. The desired developing solvent mixture is added to the chromatography tank to a depth of 0.5–1.0 cm and allowed to equilibrate.
9. Dry entire sample under nitrogen stream. Redissolve in chloroform–methanol–water (10:10:3, by vol).
10. Apply an aliquot (approx 10,000 dpm containing 10–12 µg of authentic F-Cys and GG-Cys) to the origin on the TLC plate, using a fine glass capillary or a Hamilton syringe. The addition of the unlabeled standards improves the resolution of the metabolically labeled isoprenylated cysteines.
11. Allow the sample to dry (this can be facilitated with a stream of warm air). Complete application of the labeled isoprenyl-cysteine extract to the plate in 5-µL aliquots, allowing time for the spot to dry between applications.
12. Place the plate(s) in the preequilibrated chromatography tank, and after the solvent has reached the top of each plate (1–2 h) remove and allow to air-dry in a fume hood.
13. When the plates are thoroughly dried, the radioactive zones are located with a Bioscan Imaging Scanner System 200-IBM or autoradiography (*see* **Note 11**).
14. Standard compounds are localized by exposure of the plate to the anisaldehyde spray reagent ***(32)*** or a ninhydrin spray reagent (*see* **Note 12**).

3.5. Application of These Methods to the Analysis of Individual Proteins

To illustrate the utility of this approach for individual isoprenylated proteins, these methods are applied to the analysis of a recently isolated farnesylated protein, RDJ2 (rat DnaJ homologue 2). The cDNA clone of this DnaJ-related protein was recently identified ***(36)***. The predicted amino acid sequence is found to terminate with the tetrapeptide Cys-Ala-His-Gln, which conforms to the consensus sequence for recognition by protein farnesyltransferase, and was shown to undergo farnesylation *in vivo*.

To perform this analysis, a means of specifically identifying the protein of interest must be available. In this example, a protein-specific immunoprecipitating antibody was used to isolate the protein from isotopically radiolabeled mammalian cells. However, other experimental approaches are available. (*See* **Note 13** for a discussion of these stategies.)

1. Mammalian cells are grown to near confluence and metabolically labeled with either [^{3}H]mevalonate, [^{3}H]F-OH, or [^{3}H]GG-OH as described in **Subheading 3.1**.

If a recombinant protein is to be analyzed, the mammalian cells should be transfected either stably or transiently with the expression vector prior to labeling ***(37)***.

2. The metabolically labeled cells are washed with 1–2 mL of ice-cold PBS to remove unincorporated isotopic precursor.
3. One milliliter of PBS containing 2 m*M* EDTA is added, and the cells are incubated for 5 min at room temperature. The washed cells are gently scraped from the dish with a disposable cell scraper and transferred to a 12-mL conical glass centrifuge tube.
4. The metabolically labeled cells are sedimented by centrifugation (500 *g*, 5 min), and the PBS–EDTA is removed by aspiration (avoid disrupting the cell pellet).
5. The cells are resuspended in lysis buffer (2 mL), disrupted by passage through a 20-gauge needle (3–4×), and centrifuged for 15 min at 100,000 *g* in a Beckman table top ultracentrifuge.
6. For immunoprecipitation of recombinant RDJ2, 20 µg of rabbit anti–RDJ2 antibody was added to the cleared supernatant and the mixture was incubated for 12 h at 4°C with gentle rocking (*see* **Note 14**).
7. Immune complexes were then precipitated by addition of 100 µL of a 50% slurry of protein A-Sepharose for 2 h at 4°C with gentle rocking.
8. Protein A beads were collected by centrifugation (1 min in a tabletop microfuge at 10,000 rpm). The pellet was washed 3× by resuspending in 1 mL of wash buffer A, 1 mL of wash buffer B, and 1 mL of wash buffer C, sequentially.
9. The protein is dissolved in 2% SDS, 5 m*M* β-mercaptoethanol for SDS-PAGE analysis (*see* **Subheading 3.3.** and **Fig. 6**). Alternatively, the protein A beads are subjected to pronase E digestion for analysis of isoprenyl-cysteine analysis by TLC (*see* **Subheading 3.4, Fig. 7**, and **Note 15**).

3.6. Immunodetection of AG-labeled Cellular Proteins

A recent advance in the detection of cellular isoprenylated proteins was been the generation of the unnatural F-P-P analogue AG-P-P and anti-AG antibodies ***(28)***. These reagents have been utilized to examine the prenylation status of both general cellular proteins (**Fig. 8**) and specific prenylated proteins ***(33)***. The combined use of an unnatural FPP analog and specific antibody provides a simple, inexpensive, and rapid method for monitoring both FTase activity in cells and detection of cellular prenylated proteins using non-radioactive methods (**Fig. 8**).

1. Mammalian cells are grown to near confluence and metabolically labeled with AG-OH. This can either be performed on cells treated with lovastatin (30 µ*M*) to reduce the intracellular F-P-P/GG-P-P pool and 100 m*M* AG-OH for 24 h, or cells with no lovastatin block can be incubated with 30–100 µ*M* AG-OH from 6–72 h ***(28, 33)*** (*see* **Note 16**). If a recombinant protein is to be analyzed, the mammalian cells should be transfected either stably or transiently with the expression vector prior to labeling ***(37)***.
2. The AG-OH labeled cells are washed with 1–2 mL of ice-cold PBS to remove unincorporated AG-OH, the PBS removed by aspiration, and the cells resuspended

in cell lysis buffer (300–500 μl for a 10-cm dish). The cells are disrupted by brief sonication, and protein concentration determined using Bradford reagent.

3. The AG-modified polypeptides (50–100 μg of total cell protein) can be analyzed by SDS-PAGE using an appropriate percentage polyacrylamide resolving gel (4–20%) for the proteins of interest and electrophoretically transferred to nitrocellulose membrane.
4. The membranes are incubated in PCT for 1 h at room temperature, after which the following sequential steps are performed at room temperature: incubate with polyAG-Ab (1; 5,000 dilution) in PCT for 1 h; 3 × 15 min washes with shaking in PT buffer; incubation for 1 h in PCT with goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody (dilution of 1: 20,000); 2 × 10 min washes with PT; 1 × 10 min wash with PTT; and a final 2 × 10 min wash with PT.
5. The membranes are then subjected to enhanced chemiluminescence detection by being placed in 10 mL of detection buffer (Pierce Supersignal) for 1 min and exposed to x-ray film.

4. Notes

1. Anisaldehyde spray reagent contains 10 mL of anisaldehyde, 180 mL of 95% ethanol, and 10 mL of conc. sulfuric acid, added in that order.
2. Ninhydrin spray reagent contains 0.2% ninhydrin in 95% ethanol.
3. All mammalian cells tested (CHO, C6 glioma cells and green monkey kidney [CV-1] cells) utilized F-OH and GG-OH for protein isoprenylation except murine B cells before or after activation by lipopolysaccharide (LPS). Thus, it is possible that the "salvage" pathway for F-OH and GG-OH (**Fig. 1**) may not be ubiquitous in mammalian cells.
4. The pooled organic extracts can be used for analysis of lipid products metabolically labeled by [^{3}H]F-OH (*see* ***ref. 14***). The organic solvent is evaporated under a stream of nitrogen, and the lipid residue redissolved in $CHCl_3$:CH_3OH (2:1) containing 20 μg each of authentic cholesterol and squalene. An aliquot is taken to determine the amount of radioactivity incorporated into the lipid extracts. The lipid products are analyzed on Merck silica gel G 60 TLC plates (Sigma) by developing with hexane–diethyl ether–acetic acid (65:35:1) or chloroform. Radioactive zones were located with a Bioscan Imaging Scanner System 200-IBM. Standard compounds are located with iodine vapor or anisaldehyde spray reagent (32).
5. Western transfer is preferred because transferring labeled proteins to nitrocellulose membrane appears to give a gain of 2–10-fold in sensitivity. One suspects that the polyacrylamide gel acts to quench the signal from radiolabeled protein. The transfer step serves to collect proteins in a single plane, and eliminates this problem. However, care should be taken with the intrepretation of these experiments. It is possible that some radiolabeled proteins, particularly those of either small (<10 kDa) or very large

(>100 kDa) molecular mass, may be inefficiently transferred. The properties of the protein, percentage of acrylamide, transfer buffer components, and transfer time will each influence the transfer efficiency. Direct analysis of the gel will be less sensitive, requiring more labeled protein and longer exposure times, but material will not be lost during transfer.

6. Pronase E will completely dissolve over a period of 30 min at 37°C, and these protease preparations contain sufficient esterase activity to hydrolyze the carboxymethyl esters at the C-termini of isoprenylated proteins ***(38).***
7. The lower aqueous phase remains cloudy, and contains precipitated proteins and peptides.
8. The addition of H_2O will significantly reduce the volume of the *n*-butanol phase at this step (reducing the time required for the following steps).
9. The addition of an equal volume of *n*-hexane to the *n*-butanol will speed this evaporation, by forming an azeotrope. The addition of the hexane will cause the solution to become cloudy and biphasic. The upper (butanol–hexane phase) evaporates quite rapidly, and the subsequent addition of 1 mL of 100% ethanol speeds the evaporation of the lower aqueous phase.
10. The resolution of GG-Cys and F-Cys is better in the reverse–phase chromatography system. If the normal phase system is used, the plates should be activated by heating in a 100°C oven for at least 1 h.
11. At least 5000 dpm are required for good detection using a Bioscan Imaging System, but analysis will be better (and faster) with 10,000 dpm or more. The entire sample from the digestion of 100 µg of labeled protein can usually be loaded on the TLC plates without any significant loss of chromatographic resolution.
12. Spray until plate is moist, and heat in 100°C oven until spots appear. The plates are scanned prior to spraying to avoid the reagent vapors, and because the spray reagents quench the detection of radioactivity.
13. A consideration before beginning these experiments is the abundance of the protein of interest. The analysis of a very low abundance protein will require a large number of radiolabeled tissue culture cells. Therefore, the overproduction of the protein using a mammalian expression vector may present a distinct experimental advantage. This approach also allows the cDNA to be modified to contain a unique epitope or affinity sequence at the N-terminus. In this way, proteins for which specific antibodies are not available may be studied.
14. Optimal immunoprecipitation conditions must be established for each protein-antibody complex.
15. Pronase E digestion can be carried out directly on the protein A beads without further processing. Follow directions given in **Subheading 3.4**, scaling up the volume of the proteolysis reaction mixture to provide sufficient

liquid to amply cover the protein A beads. Follow **steps 2–15** as directed. Initially, proteins should be labeled with mevalonate and both free isoprenols, to ensure correct interpretation of the results. Although a variety of proteins have been tested (*see* **Figs. 2**, **4**, and **7**) using [^{3}H]isoprenol labeling, the list of individual proteins is quite limited. It will be necessary to analyze a wide variety of defined isoprenylated proteins to further establish the reliability and limitations of this method.

16. Optimal AG-OH labeling conditions must be established for each cell culture system. Thus, pilot studies varying both the concentration (within the limits of 10–100 μM to avoid potential toxicity) and time of AG-OH labeling, in the presence or absence of lovastatin, should be performed ***(28).*** This will be particularly important if examining a specific cellular protein (*see* **Note 13**). Finally, it must be remembered that AGPP is a FTase substrate and that this approach can not be used to analyze geranylgeranylated proteins.

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PART VI

ANTIBODY TECHNIQUES

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Antibody Production

Robert Burns

1. Introduction

Antibodies are proteins that are produced by the immune systems of animals in response to foreign substances. The immune system has the ability to recognize material as nonself or foreign and mount a response to them. Substances that elicit this are known as immunogens or antigens, and one of the outcomes of the immune response is the production of antibodies that will recognize and bind to the eliciting substance.

The immune response results in the degradation and processing of the antigen by cells called macrophages and its presentation in fragments to B lymphocytes that are found in the lymphatic tissues of the animal. On presentation of the antigen fragments the B lymphocytes mature and "learn" to produce antibody molecules that have a specific affinity to the antigen fragment that they have been presented with. This process gives rise to populations of B lymphocyte clones each of which produces antibody molecules to different locations known as epitopes on the target antigen *(1)*. As individual clones produce the antibody molecules and there are many of them, the resulting antibody mix in the blood is known as polyclonal and the fluid derived from the clotted blood is known as polyclonal antiserum.

Deliberate exposure to antigens producing antibodies in animals is known as immunization, and repeated exposure leads to a condition known as hyperimmunity, in which a significantly large proportion of the circulating antibodies in the animals blood will be directed toward the antigen of interest. When exposure to the antigen ceases a stable but quiescent populations of B lymphocytes known as memory cells remain that will respond to the presence of the antigen by dividing to increase their number and the level of circulating antibody in the blood.

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The primary exposure to an antigen gives rise to a pentameric form of antibody known as immunoglobulin M (IgM). Subsequent exposure to the antigen causes maturation of the cells and a shift in the antibody type to the more stable dimeric immunoglobulin G (IgG). This shift in antibody type happens as affinity maturation of the B lymphocyte clones occurs.

Some antigens, particularly highly glycosylated proteins, do not elicit the production of memory B lymphocytes and also may not give rise to IgG antibodies even after repeated immunization. These are known as anamnestic antigens, and as no memory B lymphocytes are produced, the immune system always interprets exposure to them as a primary one and mounts an IgM response. It is possible to produce IgG to anamnestic antigens by treating them to remove the carbohydrate moieties prior to immunization, but in some cases this may lead to antibodies that do not recognize the native protein structure.

Antibodies have two qualities known as avidity and affinity. Affinity is a measure of how well the binding site on the antibody molecule fits the epitope, and antibodies, which have high affinity also, have high specificity to the target epitope. Avidity is a measure of how strong the interaction between the epitope and the antibody molecule is.

For research purposes, antibodies to the antigen of interest should be expressed at fairly high levels in the sera of donor animals, should be of high affinity, and should predominantly be composed of IgG molecules, which are stable and easily purified. This can be achieved for most antigens providing they are not too highly glycosylated, have a molecular weight >5000, and are not toxic or induce immuneparesis in the donor animal. The ability of species to respond to antigens is variable and it may be necessary to investigate which animal would be the most suitable prior to embarking on a large scale immunization project.

Monoclonal antibodies are used very successfully in many areas of research and can either replace, complement, or be used in conjunction with immunoglobulins obtained from donor serum. Monoclonal antibodies are produced by hybridoma cell lines and can be grown in tissue culture in the laboratory. Hybridomas are recombinant cell lines produced from the fusion of B cells derived from the lymphatic tissue of donor animals and myeloma cell lines that imparts immortality to the cells ***(2,3)***. As each hybridoma is descended from a single B cell clone the antibody expressed by it is of a single specificity and immunoglobulin type, and is thus termed a monoclonal antibody.

Each monoclonal antibody is monospecific and will recognize only one epitope on the antigen to which it has been raised. This may lead to practical problems if the epitope is not highly conserved on the native protein or where conformational changes may occur in the because of to shifts in pH or other environmental factors. Monoclonal antibodies are highly specific and will rarely if ever produce cross-reactions with other proteins. Polyclonal antibodies may cross-react with other closely related proteins where there are shared epitopes.

Polyclonal antisera contain a heterogeneic mixture of antibody molecules, many of which will recognize different epitopes on the protein, and their binding is much less likely to be affected by poorly conserved epitopes or changes in the protein shape.

Antisera are derived from individual animal bleeds and because of this are subject to batch variation. Individual animals can have very different immune responses to the same antigen, and individual bleeds from the same animal may vary in antibody content quite markedly. Monoclonal antibodies are produced from highly cloned cell lines that are stable and reliably produce a defined antibody product.

Polyclonal antibodies are generally less specific than monoclonals, which can be a disadvantage, as cross-reactivity may occur with nontarget proteins. Monoclonal antibodies can be too specific, as they will recognize only a single epitope that may vary on the protein of interest.

Both antibody types have their place in the research laboratory, and a careful evaluation of the required use should be undertaken before deciding which would be most applicable.

In recent years the use of non-animal-based systems have been reported and in particular the use of phage display technology for antibody production appears to be promising. Phage display libraries expressing single chain antibodies can be screened using the antigen to isolate antibodies of interest which are then expressed using vector systems such as M13 ***(4)***. These systems allow the generation of antibodies without the use of animal donors and also allow the generation of antibodies to "difficult" antigens. There are limitations in that the antibodies produced are actually antibody fragments and there may be restrictions on how they may be used in assays due to molecule size and stability. Antibodies derived by phage display technology may be transfected into cell lines such as Chinese Hamster Ovary (CHO) if large quantities of product are required. Fully human antibodies can be generated using this methodology without the use of human donor tissue and its attendant ethical concerns.

1.1. Donor Animals

Most polyclonal antibodies for research purposes are produced in domestic rabbits unless very large quantities are required, and then sheep, goats, donkeys, and even horses are used. Antibodies can also be produced in chickens; the antibodies are conveniently produced in the eggs. Rats and mice can also be used to produce antiserum but yield much smaller quantities of antibody owing to their relatively small size. Immunized mice can be used to produce 5–10 mL of polyclonal ascitic fluid by induction of ascites. Ascites is induced by the introduction of a nonsecretory myeloma cell line into the peritoneal cavity after priming with Pristane. Polyclonal antibodies are secreted into the peritoneum from the blood plasma of the animal and can achieve levels of 2–5 mg/mL. Fluid isaspirated from the peritoneum of the mouse when ascites has developed, indicated by bloating of the abdomen. The UK Home Office and other regulatory authorities now regard induction of ascites in mice as a moderate/severe procedure and this method is not normally used unless no alternative methods are available.

1.2. Adjuvants

Adjuvants are substances that increase the immune response to antigen by an animal. They may be simple chemicals such as alum, which adsorbs and aggregates proteins, increasing their effective molecular weight, or they may be specific immunestimulators such as derivatives of bacterial cell walls ***(5)***. Saponins such as Quil-A derived from the tree *Quillaja saponaria* may also be used to increase the effective immunogenicity of the antigen. Ideally an adjuvant should not induce too great an antibody response to itself to ensure that antibodies generated are specific to the antigen of interest.

The most popular adjuvant, which has been used very successfully for many years, is Freund's adjuvant. It has two formulations, complete and incomplete, which are used for the primary and subsequent immunizations, respectively. Freund's incomplete formulation is a mixture of 85% paraffin oil and 15% mannide monooleate. The complete formulation additionally contains 1 mg/mL of heat-killed *Mycobacterium tuberculosis*. In recent years the use of Freund's adjuvant has declined owing to animal welfare concerns and also the risk that it poses to workers, as it can cause localized soft tissue damage following accidental needlestick injuries. Injection preparations that contain Freund's adjuvant are also difficult to work with, as the resultant emulsion can be too thick to administer easily and it interacts with plastic syringes, preventing easy depression of the plunger.

Several adjuvant formulations are available that contain cell wall derivatives of bacteria without the intact organisms; they are much easier to administer and will achieve a similar immunostimulatory effect to Freund's without its attendant problems.

1.3. Legislation

Most countries have legislation governing the use of animals for all experimental work, and antibody production is no exception. Although the methods used are usually mild in terms of severity they must be undertaken with appropriate documentation and always by trained, authorized staff.

The legislation in terms of animal housing, immunization procedures, bleeding regimens, and choice of adjuvant vary widely according to local legislation, and it is imperative that advice be taken from the appropriate authorities prior to undertaking any antibody production work.

1.4. Antigens

The antigen chosen for an immunization program should be as close in structure and chemical identity to the target protein as possible. An exception to this is when synthetic peptides are produced to mimic parts of the native protein, an approach that is invaluable when the native antigen may be toxic to animals or nonimmunogenic.

The antigen should be soluble, stable at dilutions of approx 1 mg/mL, and capable of being administered in a liquid of close to physiological pH (6.5–7.5). The antigen should also be in as pure a form as practically possible to avoid the generation of antibodies to contaminating materials.

Many proteins are highly immunogenic in donor animals, particularly when the antigen is derived from a different species (*see* **Note 1**). Raising antibodies in the same species from which the antigen is derived from can be extremely difficult but can be overcome by conjugating the antigen to a carrier protein from another species prior to immunization. Carrier proteins such as hemoglobin, thyroglobulin, and keyole limpet hemocyanin (KLH) are commonly used. Animals immunized with the conjugated form of the antigen will produce antibodies to both the protein of interest and the carrier protein. Apart from a lower specific antibody titer in the serum there should be no interference from the carrier protein antibodies as it will not be present in the final testing system.

1.5. Test Protocol

Ideally antiserum should be tested using the procedure in which it is to be used, as antibodies may perform well using one assay but not with another. This, however, is not always practical and so a number of tests can be carried out on antiserum to test its suitability for final use. The affinity of the antiserum to the antigen can be assayed by plate-trapped double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) ***(6)***. The antigen is bound to a microtiter plate and then challenged with dilutions of the test antiserum. A secondary antispecies antibody enzyme conjugate is then added to the plate and will bind to any antibody molecules present. A chromogenic enzyme substrate is then added and the degree of color development indicates the quantity of antibody bound by the antigen.

Ouchterlony double-immunodiffusion can be used to observe the ability of the antiserum to produce immune complexes with the antigen in a semisolid gel matrix. This method can also be used to test cross-reactivity of the antiserum to other proteins closely related to the antigen. Radioimmunoassay and other related techniques can also be used to test the avidity of the antiserum to the antigen and will also give a measure of antibody titer.

In all the above tests preimmune antisera should be included to ensure that results obtained are a true reflection of antibodies produced by immunization and not due to nonspecific interactions.

2. Materials

1. At least two rabbits should be used for each polyclonal antibody production project, as they may have different immune responses to the antigen. They should be purchased from a reputable source and be parasite and disease free. New Zealand whites are often used but any of the domestic breeds can be used (*see* **Note 2**).
2. The antigen should be in a buffer of pH 6.5–7.5 and be free of toxic additives (sodium azide is often added as a preservative). A concentration of 1 mg/mL is desirable but anything above 100 µg/mL is acceptable.
3. Complete and incomplete Freund's adjuvant or any of the propriety preparations containing purified bacterial cell wall components such as Titermax and RIBI.

3. Method

1. Prior to a course of immunizations a test bleed should be taken from the rabbits to provide a source of preimmune antiserum for each animal (*see* **step 5** below). It is usual to take only 2–3 mL for the test bleeds, which will yield 1–1.5 mL of serum. The blood should be allowed to clot at 4°C for 12 h and the serum gently aspirated from the tube.
2. The antigen should be mixed with the appropriate adjuvant according to the manufacturer's instructions to achieve a final volume of 0.5 mL/injection containing 50–500 µg of antigen (*see* **Note 3**). If Freund's adjuvant is to be used, the first injection only should contain the complete formulation and the incomplete one should be used for all subsequent immunizations.
3. The rabbit should be gently restrained and the antigen–adjuvant mixture injected into the thigh muscle. Alternate legs should be used for each injection (*see* **Note 4**).
4. The immunization should be repeated 14 d after the primary one and a test bleed taken 28 d after that.
5. If the antiserum shows that the desired immune status and antibody quality has been achieved then donor bleeds can be taken. The volume of blood collected and frequency of bleeding depends on animal welfare legislation and must be adhered to. Each bleed should be assayed individually, and once the antibody titer has started to fall a further immunization can be given, followed by donor bleeds, or the animal can be terminally anesthetized and exsanguinated by cardiac puncture or by severing the carotid artery.
6. Antiserum collected from rabbits can be stored for extended periods of time at 4°C but the addition of 0.02% sodium azide is recommended to prevent adventitious bacterial growth. Antiserum quite commonly has functional antibodies even after years of refrigerated storage, but storage at –20°C is recommended for long-term preservation.
7. Antiserum will often yield in excess of 5 mg/mL of antibody and can be purified to give the immunoglobulin fraction. This can be achieved with ammonium sulfate

precipitation or by affinity chromatography using either the immobilized antigen or protein A or G. The antibody fraction can then be adjusted to 1 mg/mL, which is an ideal concentration both for its stability and for many practical applications. Purified antibody should have 0.02% sodium azide or some other preservative added to prevent the growth of adventitious organisms. Sodium azide can interfere with enzyme reactions and with photometric measurement, and this should be taken into account with regard to the final assay. Purified antibodies diluted to 1 mg/mL can be stored for long periods at 4°C with no loss of activity but for extended storage −20°C is recommended.

4. Notes

1. Some antigens will consistently fail to induce an antibody response in certain animals, and other species should be investigated as potential donors. In very rare occasions antigens will not elicit an immune response in a range of species and the nature of the antigen will then have to be investigated with a view to modifying it to increase its immunogenicity.
2. Female rabbits are less aggressive, and although smaller and yielding smaller quantities of antiserum are preferable to male rabbits for antiserum production. Many biomedical facilities use communal floor pens for donor animals and female rabbits adapt better to this form of housing.
3. The use of excessive amounts of antigen in immunizations should be avoided, as this can lead to a poor immunological response. Overloading the immune system can lead to selective deletion of the B cell clones of interest and a reduction in the specific antibody titer. High doses of antigen in the secondary and subsequent immunizations can cause anaphylactic shock and death of the donor animal.
4. It has been reported that increased stress levels in animals can depress the immune response, and appropriate measures should be taken to ensure that immunizations and bleeds are performed with the minimum of stress to the animals. General husbandry in terms of housing, noise levels, and other environmental factors should also be examined to ensure that animals for polyclonal production are maintained under suitable conditions.

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Production of Antibodies Using Proteins in Gel Bands

Sally Ann Amero, Tharappel C. James, and Sarah C. R. Elgin

1. Introduction

A number of methods for preparing proteins as antigens have been described *(1)*. These include solubilization of protein samples in buffered solutions (**ref.** ***2*** and *see* Chapter 169), solubilization of nitrocellulose filters to which proteins have been adsorbed (**ref.** ***3*** and *see* Chapter 171), and emulsification of protein bands in polyacrylamide gels for direct injections ***(4–8)***. The latter technique can be used to immunize mice or rabbits for production of antisera or to immunize mice for production of monoclonal antibodies ***(9–11)***. This approach is particularly advantageous when protein purification by other means is not practical, as in the case of proteins insoluble without detergent. A further advantage of this method is an enhancement of the immune response, since polyacrylamide helps to retain the antigen in the animal and so acts as an adjuvant *(7)*. The use of the protein directly in the gel band (without elution) is also helpful when only small amounts of protein are available. For instance, in this laboratory, we routinely immunize mice with 5–10 µg total protein using this method; we have not determined the lower limit of total protein that can be used to immunize rabbits. Since polyacrylamide is also highly immunogenic, however, it is necessary in some cases to affinity-purify the desired antibodies from the resulting antiserum or to produce hybridomas that can be screened selectively for the production of specific antibodies, to obtain the desired reagent.

2. Materials

1. Gel electrophoresis apparatus; acid-urea polyacrylamide gel or SDS-polyacrylamide gel.
2. Staining solution: 0.1% Coomassie brilliant blue-R (Sigma, St. Louis, MO, B-7920) in 50% (v/v) methanol/10% (v/v) acetic acid.

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3. Destaining solution: 5%-(v/v) methanol/7% (v/v) acetic acid.
4. 2% (v/v) glutaraldehyde (Sigma G-6257).
5. Transilluminator.
6. Sharp razor blades.
7. Conical plastic centrifuge tubes and ethanol.
8. Lyophilizer and dry ice.
9. Plastic, disposable syringes (3- and 1-mL).
10. 18-gage needles.
11. Spatula and weighing paper.
12. Freund's complete and Freund's incomplete adjuvants (Gibco Laboratories, Grand Island, NY).
13. Phosphate-buffered saline solution (PBS): 50 m*M* sodium phosphate, pH 7.25/150 m*M* sodium chloride.
14. Microemulsifying needle, 18-g.
15. Female Balb-c mice, 7–8 wk old, or New Zealand white rabbits.

3. Method

1. Following electrophoresis (*see* **Note 1**), the gel is stained by gentle agitation in several volumes of staining solution for 30 min. The gel is partially destained by gentle agitation in several changes of destaining solution for 30–45 min. Proteins in the gel are then cross-linked by immersing the gel with gentle shaking in 2% glutaraldehyde for 45–60 min ***(12)***. This step minimizes loss of proteins during subsequent destaining steps and enhances the immunological response by polymerizing the proteins. The gel is then completely destained, usually overnight (*see* **Note 2**).
2. The gel is viewed on a transilluminator, and the bands of interest are cut out with a razor blade. The gel pieces are pushed to the bottom of a conical plastic centrifuge tube with a spatula and pulverized. The samples in the tubes are frozen in dry ice and lyophilized.
3. To prepare the dried polyacrylamide pieces for injection, a small portion of the dried material is lifted out of the tube with a spatula and placed on a small square of weighing paper. In dry climates it is useful to first wipe the outside of the tube with ethanol to reduce static electricity. The material is then gently tapped into the top of a 3-mL syringe to which is attached the microemulsifying needle (**Fig. 1A**). Keeping the syringe horizontal, 200 µL of PBS solution is carefully introduced to the barrel of the syringe, and the plunger is inserted. Next, 200 µL of Freund's adjuvant is drawn into a 1-mL tuberculin syringe and transferred into the needle end of a second 3-mL syringe (**Fig. 1B**). This syringe is then attached to the free end of the microemulsifying needle. The two plungers are pushed alternatively to mix the components of the two syringes (**Fig. 1C**). These will form an emulsion within 15 min; it is generally extremely difficult to mix the material any further.
4. This mixture is injected intraperitoneally or subcutaneously into a female Balb-c mouse, or subcutaneously into the back of the neck of a rabbit (*see* **refs. *13*** and ***14***). Since the emulsion is very viscous, it is best to use 18–g needles and to anesthesize the animals. For mice, subsequent injections are administered after 2 wk and after

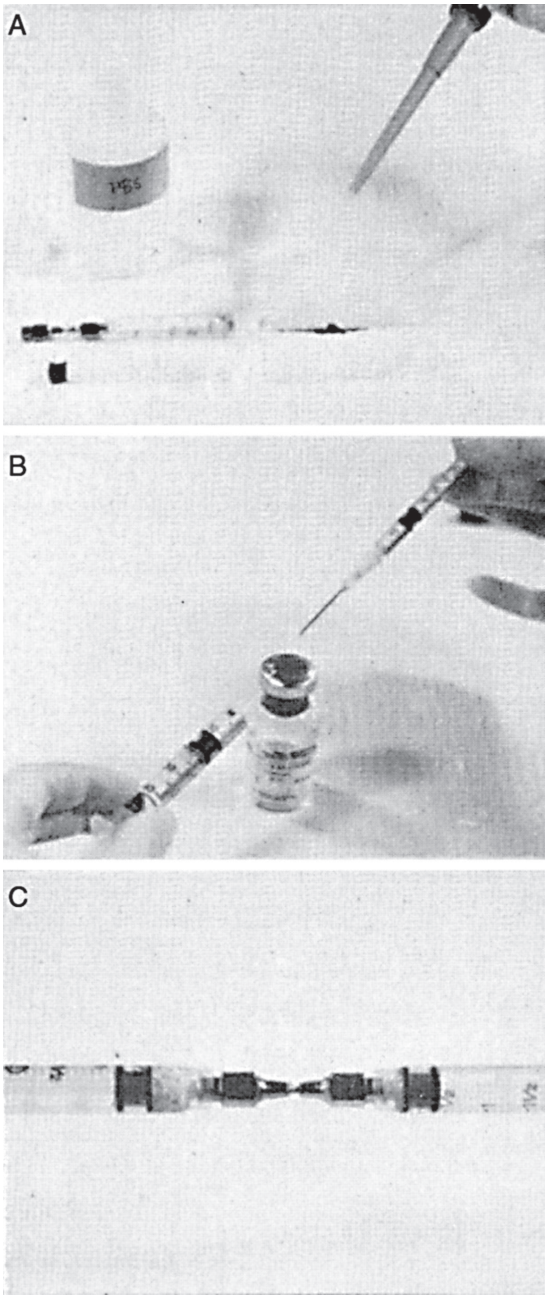

Fig. 1. Preparation of emulsion for immunizations. To prepare proteins in gel bands for injections, an emulsion of Freund's adjuvant and dried polyacrylamide pieces is prepared. **(A)** Dried polyacrylamide is resuspended in 200 µL of PBS solution in the barrel of a 3-mL syringe to which is attached a microemulsifying needle. **(B)** Freund's adjuvant is transferred into the barrel of a second 3-mL syringe. **(C)** An emulsion is formed by mixing the contents of the two syringes through the microemulsifying needle.

3 more wk. If monoclonal antibodies are desired, the animals are sacrificed 3–4 d later, and the spleen cells are fused with myeloma cells (**ref. *13***). The immunization schedule for rabbits calls for subsequent injections after 1 mo or when serum titers start to diminish. Antiserum is obtained from either tail bleeds or eye bleeds from the immunized mice, or from ear bleeds from immunized rabbits. The antibodies are assayed by any of the standard techniques (*see* Chapters 204 and 205).

4. Notes

1. We have produced antisera to protein bands in acetic acid–urea gels (*see* Chapter 27), Triton–acetic acid–urea gels ***(15, 16)*** (*see* Chapter 28), or SDS–polyacrylamide gels (*see* Chapter 21). In our experience, antibodies produced to proteins in one denaturing gel system will crossreact to those same proteins fractionated in another denaturing gel system and will usually crossreact with the native protein. We have consistently obtained antibodies from animals immunized by these procedures.
2. It is extremely important that all glutaraldehyde be removed from the gel during the destaining washes, since any residual glutaraldehyde will be toxic to the animal. Residual glutaraldehyde can easily be detected by smell. It is equally important to remove all acetic acid during lyophilization. Monoacrylamide is also toxic, whereas polyacrylamide is not. We do observe, however, that approx 50 mm^2 of polyacrylamide per injection is the maximum that a mouse can tolerate.
3. Freund's complete adjuvant is used for the initial immunization; Freund's incomplete adjuvant is used for all subsequent injections. The mycobacteria in complete adjuvant enhance the immune response by provoking a local inflammation. Additional doses of mycobacteria may be toxic.
4. High-titer antibodies have been produced from proteins in polyacrylamide gel by injecting the gel/protein mixture into the lumen of a perforated plastic golf ball implanted subcutaneously in rabbits ***(17)***. This approach places less stress on the animal, as complete adjuvants need not be used, and bleeding is eliminated. The technique has also been used in rats.

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Raising Highly Specific Polyclonal Antibodies Using Biocompatible Support-Bound Antigens

Monique Diano and André Le Bivic

1. Introduction

Highly specific antibodies directed against minor proteins, present in small amounts in biological fluids, or against insoluble cytoplasmic or membraneous proteins, are often difficult to obtain. The main reasons for this are the small amounts of protein available after the various classical purification processes and the low purity of the proteins.

In general, a crude or partially purified extract is electrophoresed on an SDS-polyacrylamide (SDS-PAGE) gel; then the protein band is lightly stained and cut out. In the simplest method, the acrylamide gel band is reduced to a pulp, mixed with Freund's adjuvant, and injected. Unfortunately, this technique is not always successful. Its failure can probably be attributed to factors such as the difficulty of disaggregating the acrylamide, the difficulty with which the protein diffuses from the gel, the presence of SDS in large quantities resulting in extensive tissue and cell damage, and finally, the toxicity of the acrylamide.

An alternative technique is to extract and concentrate the proteins from the gel by electroelution but this can lead to loss of material and low amounts of purified protein. Another technique is to transfer the separated protein from an SDS-PAGE gel to nitrocellulose. The protein-bearing nitrocellulose can be solubilized with dimethyl sulfoxide (DMSO), mixed with Freund's adjuvant, and injected into a rabbit. However, although rabbits readily tolerate DMSO, mice do not, thus making this method unsuitable for raising monoclonal antibodies.

To obtain highly specific antibodies the monoclonal approach has been considered as the best technique starting from a crude or partially purified immunogen. However, experiments have regularly demonstrated that the use of highly heterogeneous material for immunization never results in the isolation of

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clones producing antibodies directed against all the components of the mixture. Moreover, the restricted specificity of a monoclonal antibody that usually binds to a single epitope of the antigenic molecule is not always an advantage. For example, if the epitope is altered or modified (i.e., by fixative, Lowicryl embedding, or detergent), the binding of the monoclonal antibody might be compromised, or even abolished.

Because conventional polyclonal antisera are complex mixtures of a considerable number of clonal products, they are capable of binding to multiple antigenic determinants. Thus, the binding of polyclonal antisera is usually not altered by slight denaturation, structural changes, or microheterogeneity, making them suitable for a wide range of applications. However, to be effective, a polyclonal antiserum must be of the highest specificity and free of irrelevant antibodies directed against contaminating proteins, copurified with the protein of interest and/or the proteins of the bacterial cell wall present in the Freund's adjuvant. In some cases, the background generated by such irrelevant antibodies severely limits the use of polyclonal antibodies.

A simple technique for raising highly specific polyclonal antisera against minor or insoluble proteins would be of considerable value.

Here, we describe a method for producing polyclonal antibodies, which avoids both prolonged purification of antigenic proteins (with possible proteolytic degradation) and the addition of Freund's adjuvant and DMSO. Two-dimensional gel electrophoresis leads to the purification of the chosen protein in one single, short step. The resolution of this technique results in a very pure antigen, and consequently, in a very high specificity of the antibody obtained. It is a simple, rapid, and reproducible technique. Two-dimensional (2D) electrophoresis with ampholines for the isoelectric focusing (IEF) is still considered by most as time consuming and technically demanding. The introduction, however, of precast horizontal Ampholines or Immobilines IEF gels has greatly reduced this objection. Moreover, the development of 2D gel databases and the possibility to link it to DNA databases (Swiss-2D PAGE database) ***(1)*** further increases the value of using 2D electrophoresis to purify proteins of interest. Our technique was tested using two kinds of supports, nitrocellulose and polyvinylidene fluoride (PVDF), for the transfer of the protein. Both supports were tested for biocompatibility in rats and rabbits and were readily tolerated by the animals. Because PVDF is now used routinely for the analysis of proteins and peptides by mass spectrometry, it was of primary importance to test this as a prerequisite.

Even if 2D electrophoresis is a time-consuming technique it actually allows one to isolate several antigens in the same experiment, which is very important for applications such as maps of protein expression between wild-type and mutant cells or organisms or identification of proteins belonging to an isolated functional complex.

A polyclonal antibody, which by nature cannot be monospecific, can, if its titer is very high, behave like a monospecific antibody in comparison with the low titers of irrelevant antibodies in the same serum. Thus, this method is faster and performs better than other polyclonal antibody techniques while retaining all the advantages of polyclonal antibodies.

2. Materials

1. For 2D gels, materials are those described by O'Farrell ***(2,3)*** and Laemmli ***(4)***. It should be noted that for IEF, acrylamide and *bis*-acrylamide must be of the highest level of purity, and urea must be ultrapure (enzyme grade) and stirred with Servolit MB-1 from Serva (0.5 g of Servolit for 30 g of urea dissolved in 50 mL of deionized water) as recommended by Görg ***(5)***.
2. Ampholines with an appropriate pH range, for example, 5–8 or 3–9.
3. Transfer membranes: 0.45-µm BA 85 nitrocellulose membrane filters (Schleicher and Schull GmBH, Kassel, Germany); 0.22-µm membranes can be used for low molecular weight antigenic proteins.
4. Transfer buffer: 20% methanol, 150 m*M* glycine, and 20 m*M* Tris, pH 8.3.
5. Phosphate-buffered saline (PBS), sterilized by passage through a 0.22-µm filter.
6. Ponceau red: 0.2% in 3% trichloroacetic acid.
7. Small scissors.
8. Sterile blood-collecting tubes, with 0.1 *M* sodium citrate, pH 6, at a final concentration of 3.2%.
9. Ultrasonication apparatus, with 100 W minimum output. We used a IOO-W ultrasonic disintegrator with a titanium exponential microprobe with a tip diameter of 3 mm (1/8 in.). The nominal frequency of the system is 20 kc/s, and the amplitude used is 12 p.

3. Methods

This is an immunization method in which nitrocellulose-bound protein purified by 2D electrophoresis is employed and in which *neither DMSO nor Freund's adjuvant* is used, in contrast to the method described by Knudsen ***(6)***. It is equally applicable for soluble and membrane proteins.

3.1. Purification of Antigen

In brief, subcellular fractionation of the tissue is carried out to obtain the fraction containing the protein of interest. This is then subjected to separation in the first dimension by IEF with Ampholines according to O'Farrell's technique or by covalently bound Immobilines gradients. Immobilines allow a better resolution by generating narrow pH gradients (<1 pH unit), a larger loading capacity, and a greater tolerance to salt and buffer concentrations. At this point, it is important to obtain complete solubilization of the protein (*see* **Note 1**).

Separation in the second dimension is achieved by using an SDS polyacrylamide gradient gel.

The proteins are then transferred from the gel to nitrocellulose (*7*, and *see* Chapters 58–60). It is important to work with gloves when handling nitrocellulose to avoid contamination with skin keratins.

3.2. Preparation of Antigen for Immunization

1. Immerse the nitrocellulose sheet in Ponceau red solution for 1–2 min, until deep staining is obtained, then destain the sheet slightly in running distilled water for easier detection of the spots. **Never let the nitrocellulose dry out.**
2. Carefully excise the spot corresponding to the antigenic protein. Excise inside the circumference of the spot to avoid contamination by contiguous proteins (*see* **Fig. 1**).
3. Immerse the nitrocellulose spot in PBS in an Eppendorf tube (1 mL size). The PBS bath should be repeated several times until the nitrocellulose is thoroughly destained. The last bath should have a volume of about 0.5 mL, adequate for the next step.
4. Cut the nitrocellulose into very small pieces with scissors. Then rinse the scissors into the tube with PBS to avoid any loss (*see* **Fig. 2**).
5. Macerate the nitrocellulose suspension by sonication. The volume of PBS must be proportional to the surface of nitrocellulose to be sonicated. For example, 70–80 µL of PBS is adequate for about 0.4 cm^2 of nitrocellulose (*see* **Notes 3** and **4**).

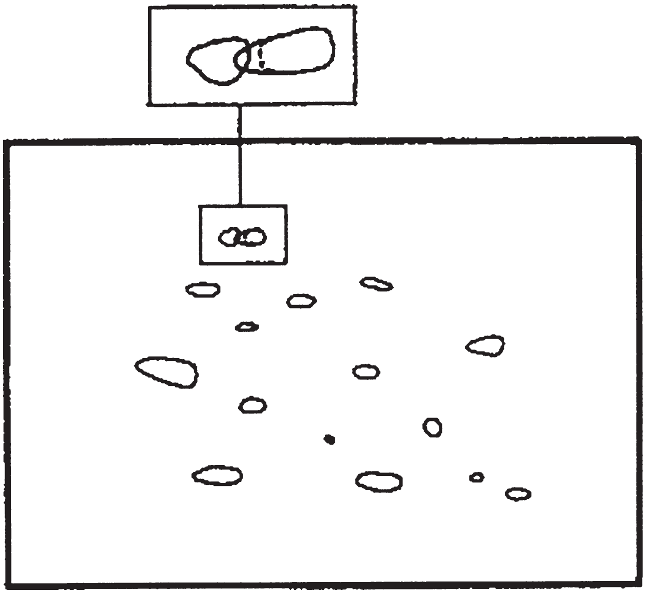

Fig. 1. Excision of the spot containing the antigen. Cut inside the circumference, for instance, along the dotted line for the right spot.

Fig. 2. Maceration of nitrocellulose.

6. After sonication, add about 1 mL of PBS to the nitrocellulose powder to dilute the mixture, and aliquot it in 500-, 350-, and 250-μL fractions and freeze these fractions at −80°C until use. Under these storage conditions, the aliquots may be used for immunization for up to 1 yr, or perhaps longer. Never store the nitrocellulose without buffer. Never use sodium azide because of its toxicity.

3.3. Immunization

1. Shave the backs of the rabbits or rats. Routinely inject two rabbits or two rats with the same antigen.
2. Thaw the 500-μL fraction for the first immunization and add 1.5–2 mL of PBS to reduce the concentration of nitrocellulose powder.

3. Inoculate the antigen, according to Vaitukaitis *(8)*, into 20 or more sites (Vaitukaitis injects at up to 40 sites). Inject subcutaneously, holding the skin between the thumb and forefinger. Inject between 50 and 100 µL (a light swelling appears at the site of injection). As the needle is withdrawn, compress the skin gently. An 18-gauge hypodermic needle is routinely used, though a finer needle (e.g., 20- or 22-gauge) may also be used (*see* **Note 5**). Care should be taken over the last injection; generally, a little powder remains in the head of the needle. Thus, after the last injection, draw up 1 mL of PBS to rinse the needle, resuspend the remaining powder in the syringe, and position the syringe vertically to inject it.
4. At 3 or 4 wk after the first immunization, the first booster inoculation is given in the same way. The amount of protein injected is generally less, corresponding to two thirds of that of the first immunization.
5. At 10 d after the second immunization, bleed the rabbit (*see* **Note 6**). A few milliliters of blood is enough to check the immune response against a crude preparation of the injected antigenic protein. The antigen is revealed on a Western blot with the specific serum diluted at 1:500 and a horseradish peroxidase-conjugated or alkaline phosphatase-conjugated second antibody. We used 3,3′ -diaminobenzidine tetrahydrochloride (DAB) for color development of peroxidase activity or the NBC-BCIP or the Western blue stabilizer (from Promega) for color development of phosphatase activity. If the protein is highly antigenic, the beginning of the immunological response is detectable.
6. At wk 2 after the second immunization, administer a third immunization in the same way as the first two, even if a positive response has been detected. If there was a positive response after the second immunization, one half of the amount of protein used for the original immunization is sufficient.
7. Bleed the rabbits 10 d after the third immunization and every week thereafter. At each bleeding, check the serum as after the second immunization, but the serum should be diluted at 1:4000 or 1:6000. Bleeding can be continued for as long as the antibody titer remains high (*see* **Note 7**). Another booster should be given when the antibody titer begins to decrease if it is necessary to obtain a very large volume of antiserum (*see* **Note 7**).
8. After bleeding, keep the blood at room temperature until it clots. Then collect the serum and centrifuge for 10 min at 3000*g* to eliminate microclots and lipids. Store aliquots at −22°C.

4. Notes

1. To ensure solubilization, the following techniques are useful:
   a. The concentration of urea in the mixture should be 9–9.5 *M*, that is, close to saturation.
   b. The protein mixture should be frozen and thawed at least 6×. Ampholines should be added only after the last thawing because freezing renders them inoperative.

   c. If the antigenic protein is very basic and outside the pH range of the Ampholines, it is always possible to carry out NEPHGE (nonequilibrium pH gradient electrophoresis) for the first dimension ***(9)***.
   d. A significant improvement using horizontal IEF is the possibility to load the sample at any place on the gel allowing to carry out in a single experiment both NEPHGE and IEF.

2. If the antigenic protein is present in small amounts in the homogenate, it is possible to save time by cutting out the part of the IEF gel where the protein is located and depositing several pieces of the first-dimension gel side by side on the second-dimension gel slab (*see* **Fig. 3**).
3. Careful attention should be paid to temperature during preparation of the antigen; always work between 2 and 4°C. Be particularly careful during sonication; wait 2–3 min between consecutive sonications. It helps to dip the eppendorf tube containing the nitrocellulose in liquid nitrogen until it is frozen and then to thaw it between each cycle of sonication.
4. This is a crucial point in the procedure. If too much PBS is added, the pieces of nitrocellulose will swirl around the probe and disintegration does not occur. In this case, the nitrocellulose pieces should be allowed to settle to the bottom of the tube before sonication and the excess buffer drawn off with a syringe or other suitable instrument (70–80 µL of PBS is sufficient for about 0.4 cm^2 of nitrocellulose). For these quantities, one or two 10-s cycles suffice to get powdered nitrocellulose. We mention the volume as a reference since the surface of nitrocellulose-bound antigen may vary.

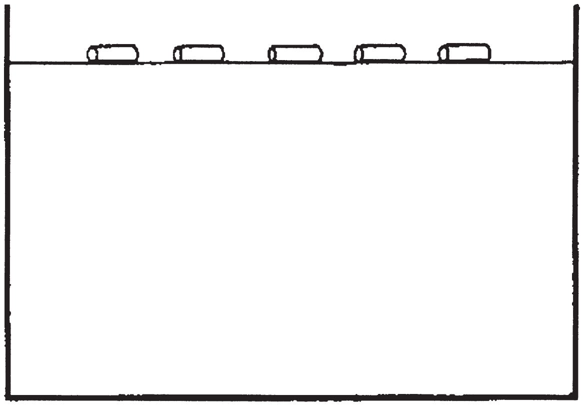

Fig. 3. Second dimension with several IEF gels. Several IEF gels are cut, 0.5 cm above and 0.5 cm below the isoelectric point of the protein of interest. They are placed side by side at the top of the second dimension slab gel. Thus, only one SDS gel is needed to collect several spots of interest.

In every case the volume of PBS must be adjusted. We experienced that PVDF is more resistant to sonication than nitrocellulose and thus several cycles of freezing and thawing are necessary.

5. What is an appropriate amount of antigenic protein to inject? There is no absolute answer to this question. It depends both on the molecular weight of the protein and also on its antigenicity. It is well known that if the amount of antigen is very low (0.5–1 µg for the classic method with Freund's adjuvant), there is no antibody production; if the amount of antigen is very high (e.g., several hundred micrograms of highly antigenic protein), antibody production might also be blocked.

   It would appear that in our method, a lower amount of antigen is required for immunization; the nitrocellulose and PVDF act as if they progressively release the bound protein, and thus, the entire amount of protein is used progressively by the cellular machinery.

   Our experiments show that a range of 10–40 µg for the first immunization generally gives good results, although, in some cases, 5 µg of material is sufficient. The nitrocellulose powder has the additional effect of triggering an inflammatory process at the sites of injection, thus enhancing the immune response, as does Freund's adjuvant by means of the emulsion of the antigenic protein with the tubercular bacillus; macrophages abound in the inflamed areas.
6. It is perhaps worth noting that careful attention should be paid to the condition of the rabbit at time of bleeding. We bleed the rabbits at the lateral ear artery. When the rabbit is calm, 80–100 mL of blood may be taken. The best time for bleeding is early in the morning, and after the rabbit has drunk. Under these conditions, the serum is very clear. It is essential to operate in a quiet atmosphere. If the rabbit is nervous or under stress, the arteries are constricted so strongly that only a few drops of blood can be obtained. Note that to avoid clotting, the needle is impregnated with a sterile sodium citrate solution by drawing the solution into the syringe 3×. The atmosphere in the animal room should be also stressless for the rats to obtain enough blood.
7. When the effective concentration required corresponds to a dilution of 1:2000, the titer is decreasing. Serum has a high titer if one can employ a dilution >1:2000 and if there is a strong specific signal without any background.
8. We have also immunized mice with nitrocellulose-bound protein by intraperitoneal injection of the powder. This is convenient when time and material are very limited, since very little protein is needed to induce a response (3–5× less than for a rabbit) and since the time lag for the response is shorter (the second immunization was 2 wk after the first, and the third

immunization, 10 d after the second). Mice have a high tolerance for the nitrocellulose powder. Unfortunately, the small amount of serum available is a limiting factor. This technique for immunizing mice can, of course, be used for the preparation of monoclonal antibodies. For people who need to raise antibodies against *Drosophila melanogaster* proteins, rats are better hosts than rabbits (mice too with the above restrictions). Nitrocellulose is injected on the top of the shoulder of the rats. The foot pad is also adequate as a site of priming injection for small quantities. Blood is collected from the tail.

9. Utilization of serum. The proper dilutions are determined. We routinely use 1:4000 for blots, 1:300–1:200 for immunofluorescence, and 1:50 for immunogold staining. Serum continues to recognize epitopes on tissue proteins after Lowicryl embedding. Labeling is highly specific, and gives a sharp image of *in situ* protein localization. *There is no need to purify the serum*. IgG purified from serum by whatever means usually gives poorer results than those obtained with diluted serum. Purification procedures often give rise to aggregates and denaturation, always increase the background, and result in loss of specific staining.
10. Bacterial antigenic protein. When antibodies are used in screening cDNA libraries in which the host is *Escherichia coli*, the antibodies produced against bacterial components of Freund's adjuvant may also recognize some *E. coli* components. An advantage of our technique is that it avoids the risk of producing antibodies against such extraneous components.
11. Is nitrocellulose antigenic? Some workers have been unable to achieve good results by immunization with nitrocellulose-bound protein. They reproducibly obtain antibodies directed against nitrocellulose. We found out that it is the result from injecting powdered nitrocellulose in Freund's adjuvant; using adjuvant actually increases the production of low-affinity IgM that binds nonspecifically to nitrocellulose. We have never observed this effect in our experiments when the technique described here was followed strictly. The same observations applied to PVDF membranes.
12. The purification step by 2D electrophoresis implies the use of denaturing conditions (SDS), and thus is not appropriate for obtaining antibodies directed against native structures. For that purpose, the protein should be transferred onto nitrocellulose after purification by classical nondenaturing methods and gel electrophoresis under nondenaturing conditions. However, it should be pointed out that, following the method of Dunn ***(10)***, it is possible partially to renature proteins with modifications of the composition of the transfer buffer.
13. Second dimension electrophoresis can be carried out with a first electrophoresis under native conditions, followed by a second electrophoresis

under denaturing conditions, that is, with SDS. Because the resolution provided by a gradient is better, it should always be used in the 2D electrophoresis. Agarose may also be used as an electrophoresis support.

14. If only a limited amount of protein is available, and/or if the antigen is weakly immunogenic, another procedure may be used. The first immunization is given as a single injection, of up to 0.8 mL, into the popliteal Iymphatic ganglion ***(10)***, using a 22-gauge needle, that is, the finest that can be used to inject nitrocellulose powder. In this case, the antigen is delivered immediately into the immune system. If necessary, both ganglions can receive an injection. The small size of the ganglions limits the injected volume. The eventual excess of antigen solution is then injected into the back of the rabbit, as described in Methods. The boosters are given in the classic manner, that is, in several subcutaneous injections into the rabbit's back. If the amount of protein available is even more limited, a guinea pig may be immunized (first immunization in the Iymphatic ganglion, and boosters, as usual).
15. The advantage of getting a high titer for the antibody of interest is that the amount of irrelevant antibodies is, by comparison, very low and, consequently, does not generate any background. Another advantage of using a crude serum with a high antibody titer is that this serum may be used without further purification to screen a cDNA expression library ***(12)***.
16. The time required for transfer and the intensity used are dependent on the molecular weight and the nature of the protein to be transferred (hydrophilic or hydrophobic). During transfer, the electrophoresis tank may be cooled with tap water. We, however, prefer semidry transfer, which is time and buffer saving and cooling is not necessary.

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Production of Antisera Using Peptide Conjugates

Thomas E. Adrian

1. Introduction

Because an immunogen requires both an antigenic site and a T-cell receptor binding site, a minimum size is necessary ***(1)***. Natural immunogens have a molecular weight >5000. Synthetic fragments of proteins may be able to bind to the surface of B cells, but do not stimulate an immune response. Such molecules are known as haptens. A hapten is an incomplete immunogen but can be made immunogenic by coupling to a suitable carrier molecule. A variety of different cross-linking agents are utilized for coupling of peptides to carrier proteins; examples of each type are covered in this chapter. In the case of larger fragments (approximately 40 amino acids), it may be possible to stimulate an immune response by presenting the peptide together with a carrier such as polyvinylpyrrolidone without the need for conjugation. This method, which is also described in this chapter, has proven to be useful for the author for a number of different peptides. Unfortunately, however, the success of this method compared with the responses to conjugated peptides is largely a matter of trial and error, and peptide fragments of this size are expensive to produce.

2. Materials

2.1. Synthetic Peptides as Haptens

There is considerable advantage to be gained by using synthetic peptides as haptens. First, it is possible to raise region-specific antibodies directed perhaps to one end, or the active site of a protein. Second, it is possible to insert particular amino acids with specific side chains for coupling. For example, a peptide can be synthesized with an extra cysteine residue at one end of the molecule to enable coupling using sulfo-SMCC ***(2)***. Alternatively, a tyrosine residue can be inserted that will enable specific coupling through the *bis*-diazotized benzidine reaction ***(3)***.

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This latter approach is valuable because the same synthetic peptide can then serve as a radioligand for metabolic studies, for receptor binding, or for a radioimmunoassay, with the assurance that the antibodies raised will not be directed toward the tyrosine residue that will be iodinated ***(3)***.

2.2. Protein Carriers

Factors governing the choice of the carrier include immunogenicity, solubility, and availability of functional groups for cross-linking. Although substances such as mucopolysaccharides, poly-L-lysine, and polyvinylpyrrolidone have been used as carriers, proteins are more widely used. Common protein carriers include serum albumin, ovalbumin, hemocyanin, and thyroglobulin. To find the very best immunogen it would be ideal to prepare conjugates with several different carriers with a range of hapten to carrier coupling ratios (*see* **Note 1**). The cost and time involved will usually make this impractical, however, and it is therefore necessary to carefully select the carrier most suitable for a particular antigen. In the classical hapten carrier system T lymphocytes recognize processed carrier determinates and cooperate with B cells which produce hapten-specific antibody response. Note that the amounts of carrier and peptide for conjugation are given in molar terms in the following conjugation protocols. This is necessary because of the wide variation in the molecular weights of potential carriers and neuropeptides to be coupled. The carrier protein represented by 100 nmol is approx 7 mg of bovine albumin, 4.5 mg of ovalbumin, 15 mg of γ-globulin, and 70 mg of thyroglobulin.

2.2.1. Bovine Serum Albumin

Because of its wide availability, high solubility, and relatively high number of coupling sites albumin is a popular choice as a carrier for weakly antigenic compounds. Albumin has a molecular weight of 67,000 and has 59 lysine residues providing primary amines useful for conjugation. A drawback in the use of albumin for conjugation is that it is frequently used in immunoassays as a non-specific blocking protein. Anti-albumin antibodies induced by its use as a carrier protein can result in false positive results in such systems.

2.2.2. Ovalbumin

Ovalbumin (egg albumin) also has wide availability, as it is the primary protein constituent of egg white. This protein is smaller than serum albumin with a molecular weight of 45,000, but contains 20 lysine residues, 14 aspartic acid, and 33 glutamic acid residues for conjugation ***(4)***. Ovalbumin exists as a single polypeptide chain with an isoelectric point of 4.6. Half of its 400 residues are hydrophobic. Caution should be exercised in handling of albumin, as it is denatured at temperatures above 56°C or even by vigorous shaking.

2.2.3. Hemocyanin

Keyhole limpit hemocyanin (KLH) is a copper-containing protein that belongs to a family of nonheme proteins found in arthropods and mollusca. KLH exists in five different aggregate states at neutral pH that will dissociate into subunits above pH 9.0 ***(5)***. KLH is a valuable carrier protein because of its large molecular mass (approx 1×10^6 to 1×10^7) and numerous lysine groups for coupling. This property of dissociation at high pH can be utilized because it increases the availability of angiogenic sites, and this can produce improved antigenic responses ***(5)***. The disadvantage of using KLH as a carrier protein is its poor water solubility. Although this makes the protein difficult to handle, it does not impair its immunogenicity.

2.2.4. Thyroglobulin

Thyroglobulin is another large molecular weight protein with a limited solubility. The advantage of thyroglobulin as a carrier comes from its large content of tyrosine residues which can be used for conjugation using the diazo reaction. The molecular weight of thyroglobulin is 670,000.

2.3. Coupling Agents

Chemical coupling agents or cross-linkers are used to conjugate small peptide haptens to large protein carriers. The most commonly used cross-linking agents have functional groups that couple to amino acid side chains of peptides (*see* **Table 1**). A host of different cross-linkers are commercially available, each with different characteristics regarding chemical groups that they are reactive toward, pH of coupling, solubility, and ability to be cleaved. The most comprehensive listing of coupling reagents is found in the Pierce catalog (Pierce, Rockford, IL; http://www.piercenet.com). This user friendly website now includes a table of all the available cross-linking agents (called: “Crosslinkers at a Glance”) and a crosslinker selection guide to identify suitable coupling agents for specific linkage sites. Several things need to be taken into consideration when selecting a bifunctional coupling reagent. First is the selection of functional groups; this can be used to produce a specific type of conjugate. For example, if the only primary amine available is in the amino-(N)-terminal end of a peptide, then a coupling agent can be selected to specifically couple in this position, leaving the carboxyl (C)-terminal end of the peptide free and available as an antigenic site. Of course, a synthetic peptide can be engineered to contain an amino acid with a specific side chain available for coupling. This is usually placed at one end of the peptide, again making the other end available as an antigenic site. Second is the length of the cross bridge; the presence of a spacer arm may make the hapten more available and therefore produce a better immune response. Third is whether the cross-linking groups are the same (homobifunctional) or different (heterobifunctional). Once again this can alter the specificity of the coupling reaction. The last consideration is whether the coupling reaction is chemical or photochemical. For a good antigenic response it is necessary to maintain the native structure of the protein complex, and this can be achieved only using mild buffer conditions and near neutral pH. The reactive groups that can be targeted using cross-linkers include primary amines, sulfhydryls, carbonyl, and carboxylic acids (*see* **Note 2**). It is difficult to predict the proximity of protein–peptide interactions. The use of bifunctional reagents with spacer arms can prevent steric hindrance and make the hapten more available for producing a good immune response.

2.3.1. Carbodiimide

Carbodiimide condenses any free carboxyl group (nonamidated C-terminal aspartate or glutamate residue) or primary amino group (N-terminal or lysyl residue), to form a peptide bond (CO–NH). The most commonly used water-soluble carbodiimide is 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (CDI or EDC). This coupling agent is very efficient and easy to use and usually couples at several alternative points on a peptide, giving rise to a variety of antigenic responses ***(6)***. Because of the unpredictable nature of the antibody responses, using this bifunctional agent the process has been termed "shotgun immunization." If it is necessary to raise antisera to a particular region of the peptide then this is not the method of choice. Furthermore, because the peptide bond linking the hapten to the carrier cannot rotate and holds the hapten physically close, steric hindrance is considerable.

Carbodiimide reacts with available carboxyl groups to form an active *O*-acylurea intermediate, which is unstable in aqueous solution, making it ineffective in two-step conjugation procedures. This intermediate then reacts with the primary amine to form an amide derivative. Failure to immediately react with an amine results with hydrolysis of the intermediate. Furthermore, hydrolysis of CDI itself is a competing reaction during the coupling and is dependent on temperature 4-morphylinoethansulfonic acid (MES) can be used as an effective carbodiimide reaction buffer in place of water. Phosphate buffers reduce the efficiency of the CDI reaction, although this can be overcome by increasing the amount of CDI used to compensate for the reduction and efficiency. Loss of efficiency of the CDI reaction is even greater with Tris, glycine, and acetate buffers, and therefore use of these these should be avoided.

2.3.2. Glutaraldehyde

Glutaraldehyde links primary amino groups (either the N-terminal or lysyl residues) on both the peptide hapten and the carrier. This linkage allows free rotation of the hapten, which reduces possible steric hindrance which may otherwise block access to the immune system by the large carrier molecule.

2.3.3. Sulfo-Succinimidyl 4-(N-maleimidomethyl) Cyclohexane-1-Carboxylate (Sulfo-SMCC)

A peptide with a free sulfhydryl group, such as a synthetic peptide with a terminal cysteine residue, provides a highly specific conjugation site for reacting with sulfo-SMCC. This cross-linker contains a maleimide group that reacts with free sulfhydryl groups, along with an *N*-hydroxysuccinimidyl ester group that reacts with primary amines ***(2)***. All peptide molecules coupled using this chemistry display the same basic antigenic conformation. They have a known and predictable orientation leaving the molecule free to interact with the immune system. This method can preserve the major epitopes on a peptide while enhancing the immune response. The water solubility of sulfo-SMCC, along with its enhanced maleimide stability, makes it a favorite for hapten carrier conjugation.

2.3.4. Bis-diazotized Benzidine

Bis-diazonium salts bridge tyrosyl or hystidyl residues between the hapten and carrier. Overnight treatment of benzidine at 4°C with nitrous acid (hydrochloric acid and sodium nitrite) results in the two amino groups being diazotized. These two diazonium groups allow coupling at both ends of the molecule. Although limited in coupling points, diazotized benzidine provides a spacer arm holding the hapten away from the carrier and usually results in an excellent antigenic response.

2.4. Adjuvants

The adjuvant is important for inducing an inflammatory response. The author has had continuous success over two decades using Freund's adjuvant. Various synthetic adjuvants are available such as AdjuPrime (Pierce) and Ribi Adjuvant System (Ribi Immunochemicals) ***(7)***. The author has had very limited success with the latter adjuvant, when used to immunize with several different small peptides. In contrast, responses with Freund's adjuvant run in parallel have always been good.

The conjugate is injected in the form of an emulsion made with Freund's adjuvant, which is a mixture of one part of detergent (Arlacel A, Sigma Chemicals) with four parts of *n*-hexadecane. This permits slow release of the coupled hapten into the circulation and may serve to protect labile antigens from degradation. Freund's adjuvant alone ("incomplete") causes an inflammatory response that stimulates antibody formation, and when made "complete" by addition of 1 mg/mL of heat-killed *Mycobacterium butyricum*, this response is further enhanced. It is convenient to purchase complete and incomplete Freund's adjuvant ready mixed (Sigma Chemicals or Calbiochem).

When preparing the emulsion, care should be taken to ensure that the oil remains in the continuous phase. Injection of aliquots of the aqueous conjugate solution into the oil via a fine bore needle, followed by repeated aspiration and ejection of the crude emulsion, will produce the required result. An alternative for making the emulsion is to use a homogenizer, although the generator should be retained specifically for this purpose to avoid subsequent peptide contamination. A simple test for the success of the preparation is to add a drop of the emulsion to the surface of water in a tube. The emulsion should stay in a single droplet without dispersing; confirming that it is immiscible and thus oil-phase continuous.

2.5. Choice of Animal for Immunization

Several factors need to be considered when choosing animal species for an immunization program, including cost, ease of handling and the volumes of antisera required (*see* **Note 3**). Small animals (such as rats and mice) have low blood volumes and present difficulties with bleeding. Large animals such as sheep or goats are expensive to house particularly over long periods. Rabbits or guinea pigs provide a near optimal solution, as they are relatively cheap to house and bleeding an ear vein or cardiac puncture in guinea pigs can provide between 10 and 30 mL of plasma from each bleed. For production of monoclonal antibodies immunization of mice is required.

3. Methods

3.1. Carbodiimide Procedure

1. Dissolve the peptide to be coupled (400 nmol) and protein carrier (100 nmol) in a small volume of water (<1 mL if possible) (*see* **Note 4**).
2. Add carbodiimide (200 mmol, 50 mg) to this solution.
3. Incubate the mixture overnight at 4°C (*see* **Notes 5** and **6**).

3.2. Glutaraldehyde Procedure

1. Dissolve the peptide (400 nmol) and carrier protein (100 nmol) in 1 mL of 0.1 *M* phosphate buffer, pH 7.5 (*see* **Note 4**).
2. Add glutaraldehyde (30 mmol, 1.5 mL of a 0.02 M solution) dropwise for 15 min.
3. Incubate the mixture overnight at 4°C (*see* **Notes 5** and **6**).

3.3. Sulfo-SMCC Procedure

1. Activate the carrier (100 nmol) by conjugating the active ester of sulfo-SMCC (2 µmol) via an amino group, in phosphate-buffered saline (PBS), pH 7.2 (with 5 m*M* EDTA) for 60 min at room temperature. This reaction results in the formation of an amide bond between the protein and the cross-linker with the release of sulfo-N-hydroxysuccinimidyl ester as a by product.

2. If desired the carrier protein can then isolated by gel filtration to remove excess reagent using a gel such as Sephadex G25 (*see* **Note 5**). Desalt by eluting the column with PBS, collecting 0.5-mL fractions. Locate the protein peak using a protein assay (Bio-Rad micro method).
3. At this stage the purified carrier possesses modifications generated by the cross-linker, resulting in a number of maleimide groups projecting from its surface. The maleimide group of sulfo-SMCC is stable for several hours in solution at physiological pH. Therefore, even after activation and purification the greatest possible activity will still be left for conjugation with the peptide.
4. The maleimide group of sulfo-SMCC reacts at pH 7.0 with free sulfhydryls on the peptide to form a stable thioether bond. The peptide (100 nmol) with a free sulfhydryl group is incubated with the maleimide-activated carrier in 1 mL of PBS (with 5 m*M* EDTA) for 2 h (or overnight if more convenient) at 4°C (*see* **Note 6**).
5. Keep sulfo-SMCC away from moisture since it is subject to hydrolysis.

3.4. Diazo Procedure

1. Freshly prepare *bis*-diazotized benzidine on each occasion in the following manner: dissolve benzidine hydrochloride (80 mmol, 20.5 mg) in 10 mL of 0.18 *M* HCl and gently mix overnight with 1 mL of 0.16 *M* $NaNO_2$ (11 mg). This reaction should take place in an ice bath inside a cold room, and the temperature should never be allowed to rise above 4°C.
2. Dissolve the peptide (400 nmol) in 130 µL (1 µmol) of fresh *bis*-diazotized benzedine solution.
3. Add $NaHCO_3$ (40 µmol, 3.4 mg) followed immediately by the addition of the carrier predissolved in a minimal volume of aqueous solution (carrier protein 100 nmol) at 4°C.
4. Adjust the pH to 9.8 with NaOH using a microelectrode.
5. Incubate the mixture overnight at 4°C (*see* **Notes 5** and **6**).

3.5. Immunization Without Conjugation to Carrier Protein

1. This procedure, using polyvinylpyrrolidone as a noncovalent carrier, can be very valuable for peptide antigens with more than 40 amino acid residues. Good results have been obtained with 30–40 amino acid peptides, but it is unlikely to produce useful antisera with smaller antigens. As well as being quick and easy, it has the added advantage of retaining tertiary peptide structure.
2. Dissolve the peptide in aqueous solution containing a 100 *M* excess of polyvinylpyrrolidone (with respect to peptide concentration).
3. Emulsify the solution in Freund’s adjuvant in the same manner as with peptide conjugates.

3.6. Making an Emulsion in Freund’s Adjuvant

1. Dissolve conjugate in water (between 10 and 100 nmol of conjugated peptide/mL, 1 mL for each rabbit being immunized).

2. Make Freund's adjuvant by mixing one part of Arlacel A with four parts of *n*-hexadecane (allowing a little more than 1 mL per rabbit).
3. For primary injections only "complete" Freund's adjuvant is used. This is made by addition of 1 mg/mL of heat-killed mycobacteria. (Boosts are given in incomplete Freund's.)
4. When preparing the emulsion, care should be taken to ensure that the oil remains in the continuous phase. Injection of aliquots of the aqueous conjugate solution into the oil via a fine bore needle, followed by repeated aspiration and ejection of the crude emulsion, will produce the required result.
5. A simple test for the success of the preparation is to add a drop of the emulsion to the surface of water in a tube. The emulsion should stay in a single droplet without dispersing, confirming that it is immiscible and thus oil-phase continuous.

3.7. Immunization Procedure

The emulsified conjugate can be administered in a variety of ways. For rabbits, the most frequently used are multiple (30–50) intradermal injections in the neck or back region, by four subcutaneous injections into each groin and axilla (0.5) *(8)*. The latter is the method we have successfully adopted for more than 20 yr. Injection into the footpads, which was at one time commonly employed, provides no advantage in terms of antibody response and should be avoided to prevent distress to the animals.

The procedure is as follows:

1. Bleed the animals and collect preimmune serum for later comparison with antisera produced by the immunization procedure.
2. Primary inoculations are given in complete Freund's adjuvant, 0.5 mL of emulsion into each groin and axilla.
3. Booster injections are given at 2–4-wk intervals, in the same manner but with incomplete Freund's adjuvant. The optimum is probably about 4 wk, but time constraints and cost may necessitate a shorter immunization schedule. With small synthetic haptens three to five, or even more boosts may be required to produce the desired high titer or high avidity antibody (*see* **Notes 7** and **8**).
4. After the first and subsequent boosts blood should be collected from an ear vein to test for the antibody titer and avidity.

3.8. Antibody Characterization for Radioimmunoassay

1. Serial dilutions of antisera are incubated with radiolabeled peptide under routine assay conditions to determine a working dilution.
2. The maximum displacement of radioactively labeled hormone from the antibody by the minimum amount of unlabeled peptide (the maximum displacement slope) is one of the main criteria for radioimmunoassay sensitivity.
3. Rapid screening for slope can be achieved by the addition of small amounts (usually about 10 fmol) of standard peptide to one set of a series of replicate antiserum dilutions set up to determine the antiserum titer. The amount of standard used should reflect the useful range (e.g., the concentration at which a hormone circulates).

4. Antibody heterogeneity may be due to use of nonhomogeneous antigens for immunization, polymerization or degradation of the hapten or carrier after coupling, or individual differences in the lymphocytic response to the antigen ***(9)***.
5. Existence of heterogeneity can be revealed by Scatchard analysis. However, for high titer antisera there is frequently effectively only a single class of antibody that predominates in the reaction. Other populations of lower concentrations and avidities make insignificant contributions.
6. Specificity should be tested using related peptides.

4. Notes

1. The ratio of hapten reactive with protein is usually arranged to be in excess of 4?1 to achieve better antigenicity with respect to the hapten. Some authorities prefer ratios as high as 40?1 but in our experience this gives a lower affinity antibody response. This is presumably due to conjugation between hapten molecules rather between hapten and carrier. The hapten and protein carriers should be both present in high concentration to increase the efficiency of the cross-linking between the molecules.
2. Coupling of a hapten at a specific site gives more chance of governing which part of the peptide becomes the antigenic determinant for the antibody, as the particular area of peptide where coupling occurs is likely to be hidden from immune surveyance.
3. Success in raising antisera is to some extent a hit and miss affair. Some workers have needed to immunize many animals to produce useful antisera, whereas three or four rabbits may produce the desired product in another immunization program. This depends, in part, on the antigenicity of the peptide and on the goals set for sensitivity and specificity. Some peptides are particularly susceptible to proteolysis (such as members of the vasoactive intestinal polypeptide family) or oxidation (such as cholecystokinin). In general, these less stable peptides make relatively poor antigens.
4. Usually the coupling agent is added after the hapten and carrier have been mixed together to minimize self-polymerization of either component.
5. Although not usually necessary, excess unreacted hapten and toxic byproducts may be removed by dialysis or gel permeation chromatography.
6. Quantification of the success of the coupling reaction is conveniently obtained by addition of a small amount of radioactively labeled hapten to the mixture prior to adding the coupling agent. Thus before and after the reaction small aliquots are removed and chromatographed. Small disposable columns contain Sephadex G-25 (Pharmacia) are ideal for this purpose. Proportion of radiation diluting in the high molecular weight position, together with the carrier, indicates the amount of coupling achieved.

7. If a particular animal has been boosted three or four times without producing detectable antibody, then the likelihood of it subsequently doing so is small and further effort is unprofitable.
8. On other occasions when animals do show a response but further boosting results in little improvement in avidity or titer, then variation in the coupling method for subsequent boosts can help. Changes including a different carrier protein or the cross-linking agent, or both, may result in production of a higher titer or more avid antisera.

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Small-Molecule–Protein Conjugation Procedures

Stephen Thompson

1. Introduction

The conjugation of small molecules (SM) to proteins is required in many areas of medical diagnostics and research. Many molecules are simply too small to elucidate an immune response, and they have to be coupled to a carrier protein such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) in order to form antigenic conjugates. Even after polyclonal (or monoclonal) antibodies have been raised to the small molecule, similar SM–enzyme conjugates are required for competitive immunoassays in which the antibodies are used to coat an enzyme-linked immunosorbent assay (ELISA) plate. Alternatively, the SM–protein conjugates can be used to coat the ELISA plate, but then the detecting antibody has to be labeled. Indeed, labeled antibodies and antigens are required in the vast majority of the diagnostic techniques discussed in this book.

It is often impossible or extremely expensive to obtain the required conjugates. This chapter therefore concentrates on the general procedures (**Subheading 3**) that can be used to couple SM carboxyl (**Subheading 3.1.1**), hydroxyl (**Subheading 3.1.2**), and amine (**Subheading 3.1.3**) residues to proteins and the analysis of the resulting conjugates. The coupling of biotin to proteins is specifically demonstrated in **Subheading 3.1.1**. Biotinylated antigens and antibodies are often used in medical diagnosis, as biotin can easily be further complexed with commercial avidin and streptavidin conjugates.

Protein–protein conjugation is sometimes required to elucidate an effective immune response. A large diagnostic antibody, marker, or protein may not be immunogeneic, as it is a natural product. However, KLH can be conjugated to it to increase its immunogenicity. Protein–protein coupling is also required to make antibody–enzyme conjugates and bispecific antibodies. A second section is therefore included that discusses antibody–KLH (**Subheading 3.2.1**),

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antibody–enzyme (**Subheading 3.2.2**), and antibody–antibody (**Subheading 3.2.3**) conjugations for the sake of completeness. Although many of the principles discussed in **Subheadings 3.1.1–3.1.3** are equally applicable to the formation of protein–protein conjugates, all such protein–protein conjugations have to be carried out in aqueous solutions to prevent denaturization/deactivation of the proteins. This second section is therefore also highly relevant in the conjugation of SM, which are only soluble in aqueous solutions to proteins.

2. Materials

1. Dry organic solvents: dioxan, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO). These are all bought over molecular sieves to minimize their water content.
2. Proteins: BSA, alkaline phosphatase (AP), KLH. (mc-KLH from Pierce is both very soluble and has a well-defined molecular weight.)
3. 1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC).
4. N-hydroxysuccinamide (NHS).
5. 1,3-Dicyclohexylcarbodiimide (DCC).
6. Biotin.
7. Carbonyldiimidazole (CDI).
8. Phosgene (Fluka).
9. p-Maleimidophenyl isocyanate (PMPI).
10. P10 desalting column (Pharmacia).
11. N-(maleimidocaproyloxy)succinimide ester (EMCS).
12. Vitamin B12.
13. Sodium dodecyl sulfate (SDS).
14. S-acetylthioglycolic acid NHS-ester (SATA).
15. 3-(2-Pyridyldithio)propionic acid NHS-ester (SPDP).

3. Methods

3.1. Small Molecule Coupling to Proteins

3.1.1. Small Molecule Carboxyl Residues

Carboxylic acid groups are normally coupled to protein amine groups using carbodiimide condensation reagents. These react with carboxylic acids to form highly reactive mixed anhydride and isourea esters ***(1,2)***. There are two major practical problems. The first is that carbodiimides are quite unstable, especially in the presence of water. The second is that the activated derivatives they form with acid groups ***(1,2)*** are even less stable, hydrolyzing in fractions of a second ***(3)***, often before you have time to add the activated SM to the protein. Luckily, most SMs (vitamins, dyes, and so on) and carbodiimides are soluble in dry organic solvents, which gets us round the first problem. The second problem can be overcome by adding NHS to the SM before the carbodiimide is added. This reacts with the SM–isourea acid derivatives as they are formed to give

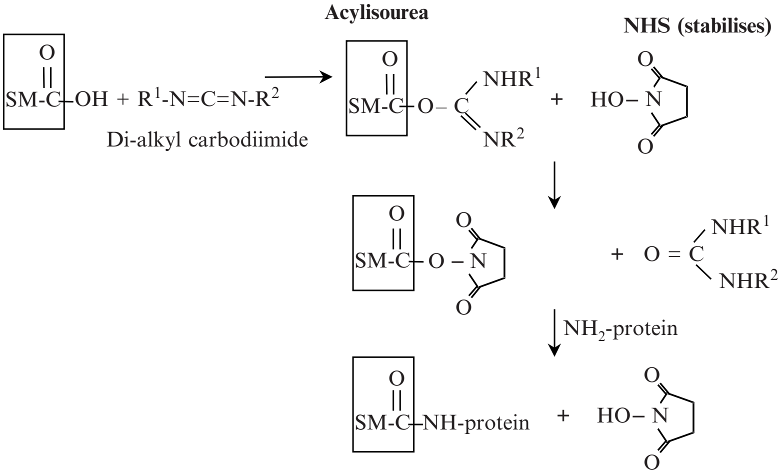

Fig. 1. The reactions involved in coupling a SM-carboxylic acid residue to a protein.

rise to activated NHS esters. These intermediates are stable indefinitely in dry conditions and have a half-life of approx 40 min in aqueous solutions ***(3)***. These activated esters can then be added to proteins in aqueous solutions and react with protein amine groups to form SM–protein amide bonds and free NHS (**Fig. 1**). If the SM is only soluble in water, the water soluble carbodiimide EDC has to be used, (normally added at twice the concentration of the SM), with the NHS added before the EDC.

A typical example of the conjugation procedure is given below using biotin, BSA, and two different carbodiimides.

3.1.1.1. BIOTIN-BSA CONJUGATION

1. Suspend 10 mg biotin in 1 mL of dry dioxan or DMF.
2. Divide into two halves and add 250 µL of NHS (10 mg/mL) to each 500-µL aliquot.
3. Add 250 µL of either EDC or DCC (both at 16 mg/mL) to each aliquot and leave for 1 h to react. As the NHS and carbodiimides are dissolved in the same dry organic solvent, this solution of activated biotin–NHS esters is stable indefinitely if kept in dry solution (*see* **Note 1**).
4. Add varying amounts of either the EDC or DCC NHS-activated biotin solutions (20, 50, or 100 µL) to 1-mL aliquots of BSA (10 mg/mL in 0.1 *M* sodium bicarbonate) and leave for 16 h to react (*see* **Note 2**).
5. Dialyze the biotin-BSA conjugates exhaustively against phosphate-buffered saline to remove uncoupled reaction products.

The formation of biotin–BSA conjugates can be confirmed by nondenaturing electrophoresis of the conjugates in 8% polyacrylamide gels (**Fig. 2, lane 4**)

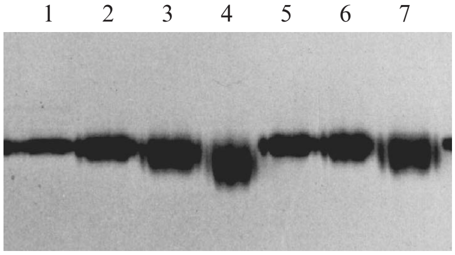

Fig. 2. The migration of BSA-Biotin conjugates in an 8% polyacrylamide gel.

All lanes contain 5 ug BSA. Lane 1 contains unconjugated BSA. Lanes 2–4 contain BSA-Biotin conjugates made when 20, 50 and 100 ul of EDC activated Biotin-NHS were added to BSA. Lanes 5–7 contain BSA-Biotin conjugates made by the addition of the same amounts of DCC activated Biotin.

using the discontinuous buffer system of Laemmli *(5)* without SDS. As more biotin is bound, the negative charge on the BSA increases (owing to loss of the positively charged NH_2 groups), and the conjugates migrate faster. Slightly more conjugation can be obtained with EDC than DCC with approx one, four, and eight residues of biotin bound to BSA in each conjugate.

It is normally fairly easy to determine how many SM residues are conjugated to a protein by comparing the OD of the substituted protein with the OD of the unsubstituted protein (**Fig. 3**). Unfortunately, biotin has no easily measured absorbance value. This is why the formation of conjugates was verified by electrophoresis. ELISA assays with labeled avidin were also used to confirm the presence of conjugates.

It is very easy to couple an SM to a protein residue via a spacer arm using this technique (*see* **Note 3**).

3.1.2. Small-Molecule Hydroxyl Residues

3.1.2.1. USING CARBONYLDIIMIDAZOLE

It is generally accepted that carbonyldiimidazole (CDI) can be used to couple hydroxyl residues to proteins in a manner similar to that discussed above for carboxylic acid residues. The CDI is added as above at an equimolar concentration to the SM in dry solvent for 2 h, and then aliquots are added to protein in 0.1 *M* sodium bicarbonate for up to 16 h. For example, dioxigenin can be bound to BSA using CDI, and it is possible to couple the light-sensitive protecting group 2-nitrobenzylalcohol to BSA *(6)*. However, the secondary alcohol, 2-nitrobenzyl ethanol, cannot be coupled to proteins by this method *(6)*. As this is a simple and cheap procedure, it should be attempted, but it will not always work.

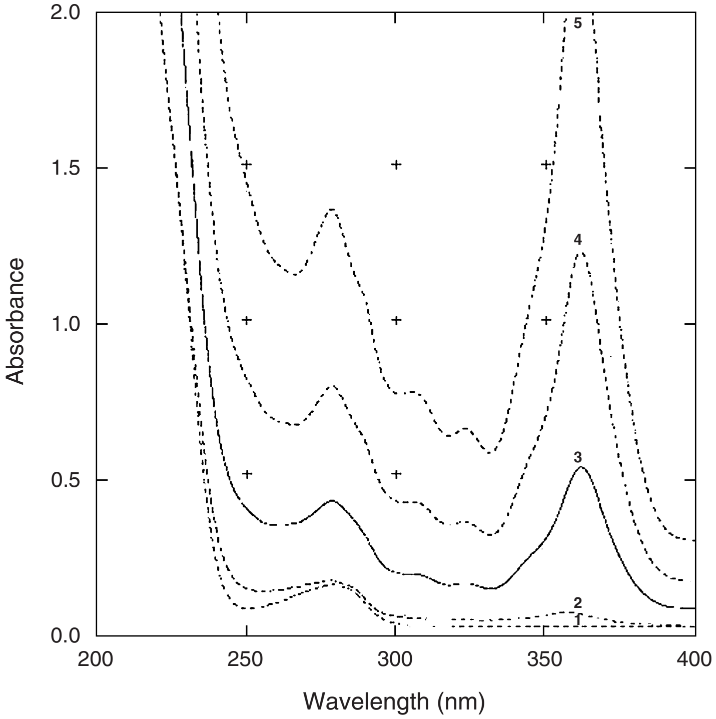

Fig. 3. Spectrophotometric scans (1–5) of Vitamin B_{12}-BSA conjugates (at 0.2 mg/ml) made by the addition of increasing amounts of Vitamin B_{12}-chloroformate (0, 1, 8, 25 and 50 mg) to 10 mg BSA.

3.1.2.2. PREDERIVITIZATION OF THE HYDROXYL RESIDUE

It is much easier to conjugate a hydroxyl group to proteins if it is first chemically modified prior to conjugation. This can easily be done by converting the hydroxyl groups to highly reactive chloroformates by treating them with equimolar amounts of diphosgene in dry dioxan in the presence of pyridine as a catalyst. The resulting chloroformates are highly reactive and spontaneously couple to protein amine groups at pH >7.5 ***(6)***. Although the chloroformates are highly reactive toward protein amine groups, they also hydrolyze rapidly in aqueous solutions. Therefore, they are normally added in large excess to the protein. A typical example of this procedure is given below in which vitamin B_{12} is coupled to BSA:

1. Suspend 84 mg of vitamin B12 in 250 µL of dry dioxan with 6 µL of pyridine and 8 µL of diphosgene. The vitamin immediately changes color from red to purple (*see* **Note 4**).

2. Leave the mixture for 15 min to react.
3. Evaporate unreacted materials.
4. Resuspend the chloroformates in 250 µL of dry solvent.
5. Add approx 1, 8, 25, and 50 mg of the B12–chloroformate to 2 mL aliquots of BSA (5 mg/mL in 0.1 *M* bicarbonate) and leave for 4 h to react.
6. Dialyze the B12–BSA conjugates for 2 d against PBS to remove uncoupled B12 and unwanted reaction products.

The pink solutions can then be measured by scanning spectrophotometry (**Fig. 3**), and the amount of vitamin coupled can be measured by the increase in absorbance at 280 nm, or more accurately by measuring the absorbance at 360 nm that was only due to B_{12}. In this case, the four conjugates were found to contain 0.1, 1.1, 2.8, and 5.2 mg of B_{12}, which represented approx 0.5, 7, 22, and 30 molecules of B_{12} bound to each BSA molecule. The covalent bonding of the vitamin groups can be confirmed by using the conjugates in competitive ELISA assays.

3.1.2.3. Utilizing a Commercial Linker Containing a Hydroxyl-Specific Isocyanate Group

There is one commercial linker, PMPI, which can be used to couple hydroxyl residues to protein sulfhydryl groups *(7)*. One end of the linker consists of an isocyanate group, which is normally first coupled at equimolar concentrations to the SM hydroxyl residue in dry organic solvents. The other end is a maleimide residue, which couples to free protein sulfhydryl groups in aqueous buffers between pH 6.5 and 7.5.

3.1.3. Small Molecule Amine Residues

SMs containing an amine residue can be coupled to proteins by simply reversing the above carbodiimide reaction. NHS is added to the protein, and it is then treated with a large excess of the water-soluble carbodiimide (EDC) to generate NHS activated carboxylic acid residues in the proteins. The NHS maintains the protein solution at around pH4.5 hence preventing the protein crosslinking to its own amine groups as they are protonated. This procedure is discussed in much greater practical detail in **Subheading 3.2.1**. The SM (dissolved in 0.1 *M* bicarbonate) is then added and left to couple for 2–16 h. After extensive dialysis, conjugation can be confirmed by spectrophotometry or non-denaturing electrophoresis. Here, however, conjugated proteins would migrate more slowly due to their loss of carboxylic acid residues.

If the SM is insoluble in water, then it can be coupled to a commercial crosslinker, which contains an NHS-activated ester at one end. This is done by simply adding the SM to the linker at an equimolar ratio for 24 h in dry DMF or DMSO. This type of linkage is also required when the protein (antibody or enzyme) loses its activity after it is treated with EDC. I have found this to happen with AP. This type of crosslinker can also be used to create a spacer arm between the SM and the protein, but as they are more commonly used to couple antibodies and enzymes together, their practical use is described below in **Subheadings 3.2.2** and **3.2.3**.

3.2. The Formation of Protein-Protein Conjugates

Protein–protein conjugates can be made by most of the procedures already discussed. However, there is one critical difference. Proteins have to be coupled in aqueous solutions in order to prevent their denaturization, precipitation, and loss of functional activity. In practical terms, this means that the organic solvents often needed to dissolve the carbodiimides and commercial linkers should never exceed 10% of the final reaction volumes.

3.2.1. Antibody–KLH Conjugates

Antibody–KLH conjugates can be used to increase the murine–murine immune response to an antibody above and beyond that obtained solely by changing the mouse strains ***(8)***. The simplest way to prepare such conjugates is to use the water-soluble carbodiimide EDC at a large molar excess to allow for the rapid hydrolysis of both the EDC and its reactive intermediates. It is now absolutely essential to include NHS in reaction mixtures prior to the addition of the carbodiimide ***(3)***. I normally treat the antibody with the carbodiimide for 15 min to activate its carboxylic acid groups and then add the activated antibody to the KLH to enable it to bind to the KLH amine groups as described below.

1. Dialyze 1 mg (0.5–3 mL) of monoclonal antibody against distilled water for at least 4 h at 0°C. This is done to remove all preservatives (e.g., azide), and buffers containing carboxylic acid and amine groups.
2. Prepare one vial of mcKLH (20 mg in 5 mL) by dialysis against 0.1 *M* bicarbonate for the same length of time.
3. Add 5 mg of NHS to the antibody followed by 2 mg EDC. Leave for 15 min to activate carboxylic acid groups.
4. Add 800 μL (approx 3 mg) of the KLH solution to the above and leave this antibody/KLH mixture to conjugate overnight. This should result in a roughly 1:1 complex given the molecular weights of IgG and KLH as 165 and 480 K, respectively.
5. Dialyze the conjugate against 0.9% saline for 24 h.

Conjugation can be confirmed by electrophoresis in SDS in 5% polyacrylamide gels (**Fig. 4**). The complexes (b and c) are at a much higher molecular weight than the KLH (a) and the monoclonal antibody (d and e; note: probably two antibodies!) starting components. Given the low intensity of staining of b and c, most of the complexes do not even penetrate the gel.

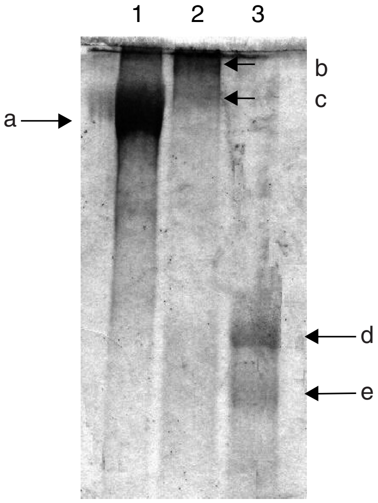

Fig. 4. The conjugation of Antibody to KLH. Lanes 1, 2, and 3 contain 15 ug mcKLH, 20 ug of antibody-KLH conjugates and 5 ug of unconjugated antibody respectively.

3.2.2. Antibody–Enzyme Conjugates

It is possible to conjugate antibodies to enzymes exactly as is given for KLH in **Subheading 3.2.1**. However, the carbodiimide-derivatized antibody can lose much of its activity. The complexes can contain several crosslinked molecules of each component! The only thing you can control is the ratio of each component to another compound. Most antibody–enzyme conjugates are therefore produced in more precise conditions using well-defined crosslinkers. The most popular consist of an NHS-ester linked to a maleimide group via a spacer arm ***(9)***. The spacer can be a benzene ring [*m*-maleimidobenzoyl-NHS ester (MBS)], cyclohexane [succinimidyl 4-(*n*-maleimido-methyl)-cyclohexane-1-carboxylate (SMCC)], or various lengths of nonaromatic carbon chain (EMCS). The selection of an appropriate spacer is very important. Rigid cyclic and aromatic spacers are themselves highly immunogeneic ***(9)***. Nonrigid carbon spacers have very little immunogenicity ***(9)***, and their flexibility can greatly enhance the sensitivity of competitive enzyme-linked immunosorbent assay (ELISAs) ***(10)*** when they are used to link SM to AP or horseradish peroxidase. NHS-esters link to primary amines at pH 7–9, whereas the maleimide groups react with sulfhydryl groups at pH 6.5–7.5. An antibody–AP conjugation procedure is given below as an example.

1. Dissolve 1–2 mg of the IgG in 1 mL 50 m*M* phosphate buffer, pH 7.0, containing 10 m*M* EDTA (*see* **Note 5**).
2. Reduce specifically at the hinge region by the addition of 2-mercaptoethylamine (6 mg in 100 µL buffer) for 90 min at 37°C.
3. Separate the reduced antibody from the reducing agent on a P10 desalting column using the same phosphate buffer containing EDTA. If 1-mL fractions are eluted,

the reduced antibody normally elutes in the fourth and fifth tubes and is stable for several hours in the presence of EDTA.

4. Dissolve one vial of AP (10,000 units, approx 4 mg) in 1 mL distilled water and prepare for coupling by dialyzing against 50 m*M* phosphate, pH 7.5, to remove amine-containing buffers and preservatives (*see* **Note 6**).
5. Dissolve 1 mg of the crosslinker (EMCS) in 100 µL DMSO and add immediately to the dialyzed AP (*see* **Note 7**).
6. Leave 30 min (no longer!) for the amine groups of the AP to couple to the NHS-ester end of the linker.
7. Remove excess unreacted linker on a P10 column.
8. Add the AP-EMCS fraction immediately to the reduced antibody and leave the final mixture for 2–16 h for the reduced antibody to react with the maleimide end of the EMCS–AP complex.
9. Dialyze to remove the EDTA from the antibody–EMCS–AP conjugate.

The antibody–EMCS–AP conjugate is checked by SDS electrophoresis in an 8% polyacrylamide gel (**Fig. 5**). Very little unreacted antibody and AP can be seen in lane 4.

3.2.3. Antibody–Antibody Conjugates

Antibody–antibody conjugates are prepared in exactly the same way as for anti-body–AP conjugates, which have just been described. Equal amounts of each antibody are used. The only problem to be considered is that one of the antibodies has to be linked via its amine groups. This can inactivate an antibody when the antibody has a susceptible amine residue in its binding region. However, a highly susceptible antibody is always reduced and linked via its hinge sulfhydryl residue to the maleimide group. It is unlikely that both antibodies would be susceptible to a loss in activity when they are linked through their amine residues. Some workers prefer to use the thiolating groups SATA ***(11)***, SPDP ***(12)***, and Trauts' reagent [iminothilane ***(13)***] to introduce sulfhydryl groups into antibodies and enzymes rather than use a reducing agent. All these agents react spontaneously with protein amine residues in aqueous solutions. Two reagents, SATA and SPDP, produce protected sulfhydryl residues, which can be released when required for crosslinking; the third directly incorporates a free SH group, but this can be unstable under certain conditions ***(14)***.

4. Notes

1. This is by far the most commonly used coupling procedure. Always use SM and carbodiimides in dry organic solvents at 1:1 molar ratios to activate the carboxyls. If the SM is only soluble in water, a large excess of EDC (around 50X) is required, and the stabilizing NHS has to be added before you add the carbodiimide.

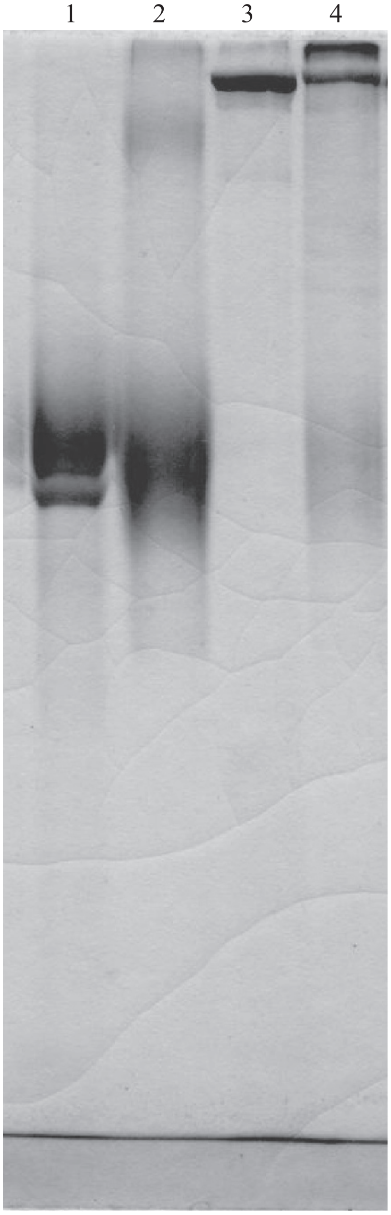

Fig. 5. The conjugation of a monoclonal antibody to AP using EMCS. Lane 1 contains AP, lane 2 contains AP treated with EMCS, lane 3 contains ed antibody and lane 4 contains the antibody-AP conjugates cross-linked by the EMCS.

2. The NHS-activated SM is always added in a large molar excess (20–50-fold) to the protein, and the extent of coupling required is discovered by trial and error. A pH > 7.0 is required to enable the activated ester to couple to the protein amine groups, as they have to be de-protonated to be able to

react. At 0.1 *M*, sodium bicarbonate has a pH of 8.3 and is a convenient and simple coupling solution.

3. It is very easy to couple an SM to a protein residue via a spacer arm using this technique. The NHS-activated SM in dry organic solvent is added to an equimolar solution of 6-aminocaproic acid (also in dry solvent) and left overnight. A peptide bond is formed with the amine residue on the caproic acid just as it would be with proteins, but here it is still in dry organic solvent. The SM acid group is now coupled via a five-carbon chain to another carboxylic acid. SM-COOH + 2HN-(CH2)5-COOH in the presence of DCC and NHS results in SM-CONH-(CH2)5-COOH. Another cycle of NHS and carbodiimide is added, and the SM–(CH2)5-CO–NHS-activated ester group is then coupled to the protein via the spacer. Alternatively, as this linker is formed in dry organic solutions, several cycles can be performed, and thus any length linker can be made, prior to the SM being coupled to the protein ***(10)***.
4. Although this is a quick and reliable method of derivatizing hydroxyl residues, diphosgene dissociates into phosgene gas, which is very dangerous. Handle very carefully in a fume hood!
5. It is very important that EDTA be present in the solutions used to reduce the antibody and in the P10 elution of the reduced antibody or it will reoxidize.
6. **It is also essential that the NHS-ester amine coupling to the enzyme be carried out at pH 7.5**. If a higher pH is used (e.g., the more normal 0.1 *M* bicarbonate buffer, pH 8.3 for 1–2 h), the maleimide group at the other end of the linker rapidly hydrolyzes. It is then impossible to bind the maleimide end of the linker to the reduced antibody in the second stage. The crosslinker maleimide group only has a half-life of 30 min at pH > 7.5. A large excess of linker at pH 7.5 and a short reaction time (20–30 min) followed by a quick separation (of unreacted linker) in a P10 column is used to reduce this problem. Lower temperatures can also be used to reduce the rate of maleimide hydrolysis. Maleimide hydrolysis while the first protein/enzyme is coupling via its amine groups is the major problem associated with maleimide-spacer-NHS-ester crosslinkers. A five-carbon straight chain linker was used in this example (EMCS), but three other linkers [MBS, SMCC, and N-(γ-maleimidobutyryloxy) succinimide ester (GMBS)] have successfully been used in an identical procedure.
7. More expensive sulfo-NHS derivatives of the above crosslinkers can be used if necessary. Their use is promoted because they are soluble in water. There are usually no problems using the normal linkers predissolved in a small amount of DMSO. However, some proteins and enzymes are extremely sensitive to the presence of organic solvents, and

the sulpho-crosslinker derivatives could then be directly substituted into the conjugation procedure.

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The Chloramine T Method for Radiolabeling Protein

Graham S. Bailey

1. Introduction

Many different substances can be labeled by radioiodination. Such labeled molecules are of major importance in a variety of investigations, e.g., studies of intermediary metabolism, determinations of agonist and antagonist binding to receptors, quantitative measurements of physiologically active molecules in tissues and biological fluids, and so forth. In most of those studies, it is necessary to measure very low concentrations of the particular substance, and that in turn, implies that it is essential to produce a radioactively labeled tracer molecule of high specific radioactivity. Such tracers, particularly in the case of proteins, can often be conveniently produced by radioiodination.

Two γ-emitting radioisotopes of iodine are widely available, ^{125}I and ^{131}I. As γ-emitters, they can be counted directly in a well-type crystal scintillation counter (commonly referred to as a γ counter) without the need for sample preparation in direct contrast to β-emitting radionuclides, such as ^{3}H and ^{14}C. Furthermore, the count rate produced by 1 g atom of ^{125}I is approx 75 and 35,000 times greater than that produced by 1 g atom of ^{3}H and ^{14}C, respectively. In theory, the use of ^{131}I would result in a further sevenfold increase in specific radioactivity. However, the isotopic abundance of commercially available ^{131}I rarely exceeds 20% owing to contaminants of ^{127}I, and its half-life is only 8 d. In contrast, the isotopic abundance of ^{125}I on receipt in the laboratory is normally at least 90% and its half-life is 60 d. Also, the counting efficiency of a typical well-type crystal scintillation counter for ^{125}I is approximately twice that for ^{131}I. Thus, in most circumstances, ^{125}I is the radionuclide of choice for radioiodination.

Several different methods of radioiodination of proteins have been developed ***(1,2)***. They differ, among other respects, in the nature of the oxidizing agent for converting ^{125}I^{-} into the reactive species ^{125}I$_2$ or ^{125}I^{+}. In the main, those reactive species substitute into tyrosine residues of the protein, but substitution into

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other residues, such as histidine, cysteine, and tryptophan, can occur in certain circumstances. It is important that the reaction conditions employed should lead on average to the incorporation of one radioactive iodine atom/molecule of protein. Greater incorporation can adversely affect the biological activity and antigenicity of the labeled protein.

The chloramine T method, developed by Hunter and Greenwood ***(3)***, is probably the most widely used of all techniques of protein radioiodination, and is used extensively for the labeling of antibodies. It is a very simple method in which the radioactive iodide is oxidized by chloramine T in aqueous solution. The oxidation is stopped after a brief period of time by addition of excess reductant. Unfortunately, some proteins are denatured under the relatively strong oxidizing conditions, so other methods of radioiodination that employ more gentle conditions have been devised, e.g., the lactoperoxidase method (*see* Chapter 175).

2. Materials

1. Na ^{125}I: 37 MBq (1 mCi) concentration 3.7 GBq/mL (100 mCi/mL).
2. Buffer A: 0.5 *M* sodium phosphate buffer, pH 7.4 (*see* **Note 1**).
3. Buffer B: 0.05 *M* sodium phosphate buffer, pH 7.4.
4. Buffer C: 0.01 *M* sodium phosphate buffer containing 1 *M* sodium chloride, 0.1% bovine serum albumin, and 1% potassium iodide, final pH 7.4.
5. Chloramine T solution: A 2 mg/mL solution in buffer B is made just prior to use (*see* **Note 2**).
6. Reductant: A 1 mg/mL solution of sodium metabisulfite in buffer C is made just prior to use.
7. Protein to be iodinated: A 0.5–2.5 mg/mL solution is made in buffer B.

3. Method

1. Into a small plastic test tube (1 × 5.5 cm) are added successively the protein to be iodinated (10 µL), radioactive iodide (5 µL), buffer A (50 µL), and chloramine T solution (25 µL) (*see* **Notes 3** and **4**).
2. After mixing by gentle shaking, the solution is allowed to stand for 30 s to allow radioiodination to take place (*see* **Note 5**).
3. Sodium metabisulfite solution (500 µL) is added to stop the radioiodination, and the resultant solution is mixed. It is then ready for purification as described in Chapter 178 and **Note 6**.

4. Notes

1. The pH optimum for the iodination of tyrosine residues of a protein by this method is about pH 7.4. Lower yields of iodinated protein are obtained at pH values below about 6.5 and above about 8.5. Indeed, above pH 8.5 the iodination of histidine residues appears to be favored.
2. If the protein is seriously damaged by the use of 50 µg of chloramine T, it may be worthwhile repeating the radioiodination using much less oxidant

(10 µg or less). Obviously, the minimum amount of chloramine T that can be used will depend, among other factors, on the nature and amount of protein to be iodinated.

3. The total volume of the reaction should be as low as practically possible to achieve a rapid and efficient incorporation of the radioactive iodine into the protein.
4. It is normal to carry out the method at room temperature. However, if the protein is especially labile, it may be beneficial to run the procedure at a lower temperature and for a longer period of time.
5. Because of the small volumes of reactants that are employed, it is essential to ensure adequate mixing at the outset of the reaction. Inadequate mixing is one of the most common reasons for a poor yield of radioiodinated protein by this procedure.
6. It is possible to carry out this type of reaction using an insoluble derivative of the sodium salt of *N*-chloro-benzene sulfonamide as the oxidant. The insoluble oxidant is available commercially (Iodo-Beads, Pierce, Rockford, IL). It offers a number of advantages over the employment of soluble chloramine T. It produces a lower risk of oxidative damage to the protein, and the reaction is stopped simply by removing the beads from the reaction mixture, thus avoiding any damage caused by the reductant.

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The Lactoperoxidase Method for Radiolabeling Protein

Graham S. Bailey

1. Introduction

This method, introduced by Marchalonis ***(1)***, employs lactoperoxidase in the presence of a trace of hydrogen peroxide to oxidize the radioactive iodide $^{125}I^-$ to produce the reactive species $^{125}I_2$ or $^{125}I^+$. These reactive species substitute mainly into tyrosine residues of the protein, although substitution into other amino acid residues can occur under certain conditions. The oxidation can be stopped by simple dilution. Although the technique should result in less chance of denaturation of susceptible proteins than the chloramine T method, it is more technically demanding and is subject to a more marked variation in optimum reaction conditions.

2. Materials

1. Na ^{125}I: 37 MBq (1 mCi) concentration 3.7 GBq/mL (100 mCi/mL).
2. Lactoperoxidase: available from various commercial sources. A stock solution of 10 mg/mL in 0.1 *M* sodium acetate buffer, pH 5.6, can be made and stored at −20°C in small aliquots. A working solution of 20 µg/mL is made by dilution in buffer just prior to use.
3. Buffer A: 0.1 *M* sodium acetate buffer, pH 5.6 (*see* **Note 1**).
4. Buffer B: 0.05 *M* sodium phosphate buffer containing 0.1% sodium azide, final pH 7.4.
5. Buffer C: 0.05 *M* sodium phosphate buffer containing 1 *M* sodium chloride 0.1% bovine serum albumin and 1% potassium iodide, final pH 7.4.
6. Hydrogen peroxide: A solution of 10 µg/mL is made by dilution just prior to use.
7. Protein to be iodinated: A 0.5–2.5 mg/mL solution is made in buffer A.

It is essential that none of the solutions except buffer B contain sodium azide as antibacterial agent, since it inhibits lactoperoxidase.

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3. Method

1. Into a small plastic test tube (1 × 5.5 cm) are added, in turn the protein to be iodinated (5 µL), radioactive iodide (5 µL), lactoperoxidase solution (5 µL), and buffer A (45 µL).
2. The reaction is started by the addition of the hydrogen peroxide solution (10 µL) with mixing (*see* **Note 2**).
3. The reaction is stopped after 20 min (*see* **Note 3**) by the addition of buffer B (0.5 mL) with mixing.
4. After 5 min, buffer C (0.5 mL) is added with mixing. The solution is then ready for purification as described in Chapter 178 (*see* **Note 4**).

4. Notes

1. The exact nature of buffer A will depend on the properties of the protein to be radioiodinated. Proteins differ markedly in their pH optima for radioiodination by this method ***(2)***. Obviously the pH to be used will also depend on the stability of the protein, and the optimum pH can be established by trial and error.
2. Other reaction conditions, such as amount of lactoperoxidase, amount and frequency of addition of hydrogen peroxide, and so forth, also markedly affect the yield and quality of the radioiodinated protein. Optimum conditions can be found by trial and error.
3. The longer the time of the incubation, the greater the risk of potential damage to the protein by the radioactive iodide. Thus, it is best to keep the time of exposure of the protein to the radioactive iodide as short as possible, but commensurate with a good yield of radioactive product.
4. Some of the lactoperoxidase itself may become radioiodinated, which may result in difficulties in purification if the enzyme is of a similar size to the protein being labeled. Thus, it is best to keep the ratio of the amount of protein being labeled to the amount of lactoperoxidase used as high as possible.

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The Bolton and Hunter Method for Radiolabeling Protein

Graham S. Bailey

1. Introduction

This is an indirect method in which an acylating reagent (*N*-succinimidyl-3 [4-hydroxyphenyl]propionate, the Bolton and Hunter reagent), commercially available in a radioiodinated form, is covalently coupled to the protein to be labeled ***(1)***. The [^{125}I] Bolton and Hunter reagent reacts mostly with the side-chain amino groups of lysine residues to produce the labeled protein. It is the method of choice for radiolabeling proteins that lack tyrosine and histidine residues, or where reaction at those residues affects biological activity. It is particularly suitable for proteins that are sensitive to the oxidative procedures employed in other methods.

2. Materials

1. [^{125}I] Bolton and Hunter reagent: 37 MBq (1 mCi), concentration 185 MBq/mL (5 mCi/mL) (*see* **Note 1**).
2. Buffer A: 0.1 *M* sodium borate buffer, pH 8.5.
3. Buffer B: 0.2 *M* glycine in 0.1 *M* sodium borate buffer, pH 8.5.
4. Buffer C: 0.05 *M* sodium phosphate buffer containing 0.25% gelatin.
5. Protein to be iodinated: A 0.5–2.5 mg/mL solution is made in buffer A.

It is essential that none of the solutions contain sodium azide or substances with free thiol or amino groups (apart from the protein to be labeled), since the Bolton and Hunter reagent will react with those compounds.

3. Method

1. The [^{125}I] Bolton and Hunter reagent (0.2 mL) is added to a small glass test tube (1 × 5.5 cm) and is evaporated to dryness under a stream of dry nitrogen.
2. All reactants are cooled in iced water (*see* **Note 2**).

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3. The protein to be iodinated (10 µL) is added, and the tube is gently shaken for 15 min (*see* **Note 2**).
4. Buffer B (0.5 mL) is added (*see* **Note 3**). The solution is mixed and allowed to stand for 5 min.
5. Buffer C (0.5 mL) is added with mixing (*see* **Note 4**). The resultant solution is then ready for purification.

4. Notes

1. [^{125}I] Bolton and Hunter reagent is available from Amersham International (Little Chalfort, UK) and Dupont NEN (Stevenage, UK). The Amersham product is supplied in anhydrous benzene containing 0.2% dimethylformamide. Aliquots can be easily withdrawn from the vial. However, the Dupont NEN is supplied in dry benzene alone, and dry dimethylformamide (about 0.5% of the sample volume) must be added to the vial with gentle shaking to facilitate the removal of aliquots.
2. [^{125}I] Bolton and Hunter reagent is readily hydrolyzed in aqueous solution. Under the described conditions, its half-life is about 10 min.
3. Buffer B stops the reaction by providing an excess of amino groups (0.2 *M* glycine) for conjugation with the [^{125}I] Bolton and Hunter reagent. Thus, the carrier protein (0.25% gelatin) in buffer C does not become labeled.
4. This method of radioiodination has been used extensively, and various modifications of the described procedure, including time and temperature of the reaction, have been reported ***(2)***. For example, it is possible first to acylate the protein with the Bolton and Hunter reagent, and then carry out radioactive labeling of the conjugate using the chloramine T method. However, in general, this procedure does not seem to offer any advantage over the method described here. Also, the time and temperature of the reaction can be altered to achieve optimal labeling.

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Preparation of ^{125}I-Labeled Peptides and Proteins with High Specific Activity Using IODO-GEN

J. Michael Conlon

1. Introduction

The reagent IODO-GEN (1,3,4, 6-tetrachloro-3α, 6α-diphenylglycoluril) was first introduced by Fraker and Speck in 1978 ***(1)*** and rapidly found widespread use for the radioiodination of both peptides and proteins ***(2)***. Although the usual application of the reagent in the laboratory is for introduction of an atom of ^{125}I, IODO-GEN has been used successfully for the preparation of proteins, particularly monoclonal antibodies, labeled with ^{131}I^{124} and ^{123}I for use in immunoscintigraphy and positron emission tomography ***(3–5)***. This chapter addresses only the use of IODO-GEN to radiolabel pure peptides/proteins in aqueous solution, but iodination of proteins on the cell surface of a wide range of intact eukaryotic and prokaryotic cells ***(6,7)*** and on subcellular organelles ***(8)*** and plasma lipoproteins ***(9)*** has been accomplished using the reagent. The advantages of IODO-GEN over alternative reagents previously used for radiolabeling such as chloramine T, lactoperoxidase/hydrogen peroxide, and ^{125}I-labeled Bolton–Hunter reagent (*N*-succinimidyl-3 [4-hydroxyphenyl] propionate) are that the reaction is rapid, technically simple to perform, and gives reproducibly high levels of incorporation of radioactivity with minimal oxidative damage to the protein.

The structure of IODO-GEN is shown in **Fig. 1**. The reagent is virtually insoluble in water and so a solution in dichloromethane or chloroform is used to prepare a thin film of material on the walls of the reaction vessel. Addition of an aqueous solution of Na^+ ^{125}I generates the reactive intermediate (I^+/I_3^+) that participates in electrophilic attack primarily at tyrosine but also at histidine residues in the peptide/protein. Reaction is terminated simply by transferring the contents of the reaction vessel to a clean tube, thereby obviating the need to add a reducing agent. In comparison to other radio-iodination methods, particularly the use of chloramine T and lactoperoxidase, oxidative damage to sensitive

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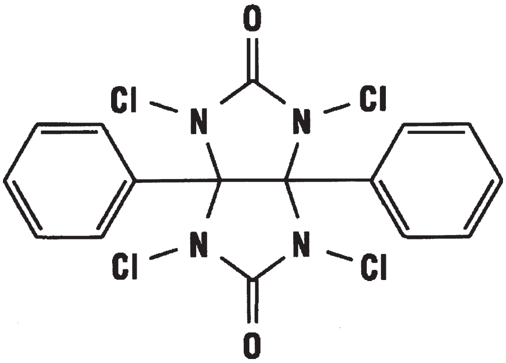

Fig. 1. The structure of IODO-GEN (1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril).

residues in the peptide/protein (particularly methionine and tryptophan) is much less using IODO-GEN ***(10)***. To minimize oxidative damage even further and to limit production of diiodotyrosyl derivatives, the strategy of trace labeling is recommended. This involves radioiodination of only approx 10% of the molecules and necessitates the separation, as completely as possible, of the radiolabeled peptide from the unreacted starting material in order to obtain a tracer of sufficiently high specific activity to be of use in radioimmunoassay, radioreceptor assay, or autoradiography. Reversed-phase high-performance liquid chromatography (RP-HPLC) combines rapidity and ease of operation with optimum separation of labeled and unlabeled peptide (reviewed in ***(11)***). The availability of wide-pore C_3 and C_4 columns generally permits good recoveries of labeled proteins with molecular mass (M_r) > 10,000. Techniques such as selective adsorption to diatomaceous materials (e.g., talc or microfine silica) and gel permeation chromatography give tracers of low specific activity, and ion-exchange chromatography is time consuming and results in a sample dilution that may be unacceptable.

Recent applications of IODO-GEN in the field of protein and peptide radioiodination involve the use of the reagent in the preparation of N-succinimidyl 4-guanidinomethyl-3-[^{131}I]iodobenzoate ***(12)***, N-succinimidyl 3-[^{131}I]iodo-4-phosphonomethylbenzoate ***(13)***, and N-succinimidyl-5-[^{131}I]iodopyridine-3-carboxylate ***(14)*** as superior conjugation alternatives to the Bolton–Hunter reagent, particularly for labelling monoclonal antibodies.

2. Materials

2.1. Apparatus

No specialized equipment is required to carry out the iodination, but the reaction should be carried out in an efficient fume hood. Reaction takes place in a 1.5-mL natural-colored polypropylene microcentrifuge (Eppendorf tube) (*see* **Note 1**), immersed in an ice bath. A nitrogen or argon cylinder is required for removal of solvent. Liquids are dispensed with 10- and 100-μL pipets (e.g., Gilson Pipetman) that, because of inevitable contamination by radioactivity, should be dedicated to the reaction and stored in a designated area.

For HPLC, a system capable of generating reproducible linear two solvent gradients is required. Again, because of contamination by radioactivity, an injector, for example, Rheodyne Model 7125 with 1-mL sample loop; a 1-mL leak-free injection syringe, for example, Hamilton Gastight (cat. no. 1001); a 25 × 0.46 cm analytical reversed-phase column (*see* **Note 2** on column selection); and a fraction collector capable of collecting a minimum of 70 samples, for example, Frac 100 (Pharmacia, Uppsala, Sweden) should be dedicated to the radioiodination procedure. A UV detection system and chart recorder/integrator are not necessary unless it is important to demonstrate that the radiolabeled component has been completely separated from the starting material.

2.2. Chemicals

2.2.1. Iodination Reagents

1. IODO-GEN (Pierce, Rockford, IL): The reagent should be stored in a desiccator in the freezer and the bottle protected from light.
2. Dichloromethane (stabilized, HPLC grade): Redistillation is not required.
3. Carrier-free $Na^+ \, ^{125}I^-$ in 0.1 *M* NaOH (3.7 GBq/mL; 100 mCi/mL, Amersham Pharmacia Biotech, Piscataway, NJ).
4. 0.2 *M* Disodium hydrogen phosphate–sodium dihydrogen phosphate buffer, pH 7.5 (*see* **Note 3**).

2.2.2. Chromatography

1. Solvent A: Add 1 mL of trifluoroacetic acid (Pierce HPLC/Spectro grade) to 1000 mL of water (*see* **Note 4**).
2. Solvent B: Mix 700 mL of acetonitrile (Fisher optima grade) with 300 mL of water and add 1 mL of trifluoroacetic acid. The solvents should be degassed, preferably by sparging with helium for 1 min, but passage through a filter is unnecessary.

3. Method

3.1. Radioiodination

1. Dissolve 1.5 mg of IODO-GEN in 2 mL of dichloromethane.
2. Pipet 20 μL of the solution into a polypropylene tube and remove the solvent in a gentle stream of nitrogen or argon at room temperature. The aim is to produce a film of IODO-GEN on the wall of the tube. If the reagent has formed a visible clump, the tube should be discarded and a new tube prepared. According to the manufacturer's instructions (Pierce), the tubes can be stored in a vacuum desiccator for up to 2 mo, but it is recommended that a tube is prepared freshly for each reaction. The tube is set in an ice bath for 10 min prior to the iodination (*see* **Note 5**).

3. Dissolve 10 nmol of the peptide (*see* **Note 6**) in 0.2 *M* sodium phosphate buffer, pH 7.5 (100 µL) and pipet the solution into the chilled IODO-GEN-coated tube.
4. Add the Na^+ $^{125}I^-$ solution (5 µL; 0.5 mCi or 10 µL; 1 mCi depending on the quantity of tracer required).
5. Allow the reaction to proceed for between 1 and 20 min (*see* **Note 7**). The contents of the tube should be gently agitated by periodically tapping the side of the tube with a gloved finger.
6. Reaction is stopped by aspirating the contents of the tube into a solution of 0.1% (v/v) trifluoroacetic acid–water (500 µL) contained in a second polypropylene tube. The reaction vessel may be washed with a further 200 µL of trifluoroacetic acid–water (500 µL) contained in a second polypropylene tube. The reaction vessel may be washed with a further 200 µL of trifluoroacetic acid–water and the washings combined with the reaction mixture.

3.2. Chromatography

1. Prior to carrying out the iodination, the column is equilibrated with 100% solvent A at a flow rate of 1.5 mL/min for at least 30 min. In the case of more hydrophobic peptides/proteins, the column is equilibrated with starting solvent containing up to 30% solvent B (21% acetonitrile).
2. The instrument is programmed to increase the proportion of solvent B from starting conditions to 70% (49 % acetonitrile) over 60 min using a linear gradient (*see* **Note 8**).
3. The reaction mixture is injected onto the column (*see* **Note 9**). The fraction collector, programmed to collect 1-min fractions, is started and the linear elution gradient is begun. A total of 60 fractions are collected.
4. At the end of the chromatography, the column is irrigated with 100% solvent B for 30 min. The column may be stored in this solvent.
5. The radioactivity in aliquots (2 mL) of each fraction is counted in a gamma scintillation counter (*see* **Note 10** for optimum storage conditions of tracer).

The results of a typical radioiodination are illustrated in **Fig. 2**. The reaction mixture comprised 10 nmol of rat galanin and 0.5 mCi of Na^+ $^{125}I^-$ and the reaction time was 2 min. Unreacted free iodide was eluted at the void volume of the column (fractions 5 and 6). The fraction denoted by the bar (tube 51) was of high specific activity (approx 74 TBq/mmol; 2000 Ci/mmol). The earlier eluting minor peak of radioactivity (tube 49) probably represented the diiodotyrosyl derivative. Before use in radioimmunoassay or radioreceptor studies, the quality of the tracer is assessed by incubating an aliquot (approx 20,000 cpm) with an excess of an antiserum raised against galanin (1 : 1000 dilution) in 0.1 *M* sodium phosphate buffer, pH 7.4 (final volume 300 µL) for 16 h at 4°C. Free and bound radioactivity are separated by addition of 100 µL of a 10 mg/mL solution of bovine γ-globulin (Sigma, St. Louis, MO) and 1 mL of 20% (w/v) solution of polyethylene glycol 6000 (Sigma, approx M_r 8000) in water followed by centrifugation (1600*g* for 30 min at 4°C). Under these conditions, >90% of the radioactivity is bound to antibody.

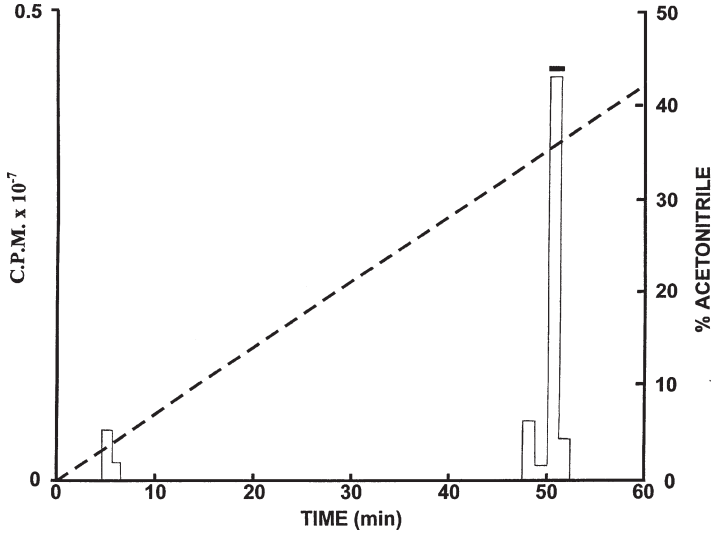

Fig. 2. RP-HPLC on a (0.46 × 25 cm) Vydac 218TP54 (C_{18}) column of the reaction mixture following incubation of 10 nmol of rat galanin with 0.5 mCi of Na ^{125}I in an IODOGEN-coated tube for 2 min at 0°C. Fractions (1 min) were collected and the fraction denoted by the *bar* contained tracer of high specific activity (74 TBq/mmol). The *dashed line* shows the concentration of acetonitrile in the eluting solvent.

4. Notes

1. A systematic comparison of the properties of different IODO-GEN-coated surfaces has concluded that soda-lime glass produced the most rapid rate of oxidation of Na ^{125}I ***(15)***. However, irreversible binding of most peptides to the walls of polypropylene tubes is much less than to glass and so clear polypropylene Eppendorf tubes are routinely used in the author's laboratory. The use of borosilicate glass tubes ***(15)*** and polysytrene tubes is not recommended. Glass test tubes (12 mm × 75 mm) precoated with IODO-GEN (50 µg) are available from Pierce.
2. The choice of column is dictated by the nature of the radiolabeled peptide to be purified. For relatively small (M_r < 3000) peptides, good resolution and recoveries are generally obtained with (0.46 × 25 cm) narrow pore (80 Å), 5-µm particle size octadecylsilane (C_{18}) columns such as Supelcosil LC-18-DB (Supelco, Bellefonte, PA), Ultrasphere ODS (Beckman, Duarte, CA), or Spheri-5 RP-18 (Brownlee/Perkin Elmer, Foster City, CA). For purification of radiolabeled tracers of higher molecular mass (M_r > 3000), the use of columns containing wide-pore (300 Å) 5-µm particle size C_{18} packing materials is recommended. Suitable columns include

Vydac 218TP54 (Separations Group, Hesperia, CA), Spherisorb wide-pore C_{18} (Waters Corporation, U. K.), Waters Delta-Pak C_{18} and Ultrapore C_{18} (Beckman). For purification of tracers of molecular mass > 10,000, such as the pituitary glycoprotein hormones, sharper peaks and better recoveries of radioactivity may be obtained using wide-pore silica with propyl and butyl carbon loading. Suitable columns include Beckman Ultrapore C_3 and Vydac 214TP54 C_4 columns.

3. The reaction is not markedly pH sensitive and high incorporations of ^{125}I may be achieved in the pH range 6–9 ***(16)***.
4. Suitable water can be obtained using a Milli-Q purification system (Millipore) supplied with water that has been partially purified by single distillation or by treatment with a deionization resin.
5. A low reaction temperature is important in minimizing damage to the radiolabeled peptide/protein. For example, the use of IODO-GEN at room temperature to prepare ^{125}I-labeled human growth hormone resulted in the production of tracer containing a significant amount of the hormone in an aggregated form whereas only the radio-labeled monomer was produced when the reaction was carried out at 0°C ***(17)***.
6. As many peptides are relatively insoluble in buffers of neutral pH, it is recommended that the peptide first be dissolved in a minimum volume (approx 5 µL) of 0.1% (v/v) trifluoroacetic acid/water and the volume made up to 100 µL with 0.2 *M* sodium phosphate buffer, pH 7.5.
7. The optimum reaction time must be determined for each peptide and protein, but some general guidelines can be given. For small peptides (<15 amino acid residues) containing a tyrosine residue in a sterically unhindered region of the molecule, for example, the N- or C-terminal residue, the reaction proceeds rapidly and reaction times of between 0.5 and 2 min are generally sufficient. For larger peptides and proteins, it is often necessary to prolong the reaction time to between 10 and 15 min. Peptides that do not contain a tyrosine residue but possess a sterically unhindered histidine (e.g., neurokinin B ***(18)*** and secretin) may be iodinated using IODO-GEN, but longer reaction times (up to 20 min) may be required.
8. The relatively steep gradient (0 → 70% solvent B, equivalent to 0 → 49% acetonitrile) over 60 min is recommended as the initial elution conditions when preparing a radiolabel for the first time. Better separation of the tracer and the unlabeled peptide will be obtained using a shallower gradient. For example, relatively hydrophilic peptides such as [Tyr^0]bradykinin and [Tyr^8]substance P ***(19)*** may be purified using a gradient of 0 → 35% acetonitrile over 60 min, whereas hydrophobic peptides such as corticotropin-releasing hormone, urotensin-1, and neurokinin B may be purified using a gradient of 21 → 49% acetonitrile over 60 min. In a case where

separation of radiolabeled peptide and starting material is incomplete, a label of higher specific activity may be obtained by prolonging the time of chromatography and collecting smaller fractions.

9. In some published protocols, the radiolabeled peptide or protein and unreacted ^{125}I- are separated prior to RP-HPLC, for example, by adsorption on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) or by gel-permeation chromatography on a Sephadex G-10 desalting column (Pharmacia). This procedure is not necessary and it is recommended that the reaction mixture is injected directly onto the HPLC column.
10. The stability of radiolabeled peptides and proteins varies dramatically, with useful lives ranging from a few days to more than 2 mo. Repeated freezing and thawing of the tracer is not recommended and so the HPLC fraction(s) containing the radiolabel should be aliquoted immediately, diluted with one volume ethanol or methanol, and stored at as low a temperature as possible (–70°C is preferred). The volume of the aliquot should be related to the size of a typical assay.
11. Although IODO-GEN is almost insoluble in water, its solubility in buffers containing detergent increases appreciably. Under these circumstances, oxidative damage to the peptide/protein may occur and the use of an alternative reagent IODO-BEADS (Pierce) should be considered. IODO-BEADS comprise the sodium salt of *N*-chloro-benzenesulfonamide immobilized on nonporous polystyrene beads ***(20)***. The reaction conditions and purification protocol using IODO-BEADS are the same as using IODO-GEN except that one or more of the beads are substituted for the film of IODO-GEN. High incorporations of radio-activity are observed even in the presence of detergents or chaotropic reagents, for example, urea.

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Purification and Assessment of Quality of Radioiodinated Protein

Graham S. Bailey

1. Introduction

At the end of a radioiodination procedure, the reaction mixture will contain the labeled protein, unlabeled protein, radioiodide, mineral salts, enzyme (in the case of the lactoperoxidase method), and possibly some protein that has been damaged during the oxidation. For most uses of radioiodinated proteins, it is essential to have the labeled species as pure as possible. In theory, any of the many methods of purifying proteins can be employed ***(1)***. However, the purification of the radioiodinated protein should be achieved as rapidly as possible. For that purpose, the most widely used of all separation techniques is gel filtration.

One of the most important parameters used to assess the quality of a purified labeled protein is its specific radioactivity, which is the amount of radioactivity incorporated/unit mass of protein. It can be calculated in terms of the total radioactivity employed, the amount of the iodination mixture transferred to the gel-filtration column, and the amount of radioactivity present in the labeled protein, in the damaged components, and in the residual radioiodine. However, in practice, the calculation does not usually take into account damaged and undamaged protein. The specific activity is thus calculated from the yield of the radioiodination procedure, the amount of radioiodide, and the amount of protein used, assuming that there are no significant losses of those two reactants. The yield of the reaction is simply the percentage incorporation of the radionuclide into the protein.

It is obviously important that the radioiodinated protein should as far as possible have the same properties as the unlabeled species. Thus, the behavior of both molecules can be checked on electrophoresis or ion-exchange chromatography.

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The ability of the two species to bind to specific antibodies can be assessed by radioimmunoassay.

This chapter will describe a protocol for the purification of a radiolabeled protein and an example of a calculation of specific radioactivity.

2. Materials

1. Sephadex G75 resin.
2. Buffer A: 0.05 *M* sodium phosphate containing 0.1% bovine serum albumin and 0.15 *M* sodium chloride, final pH 7.4.
3. Specific antiseum to the protein.
4. Buffer B: 0.1 *M* sodium phosphate buffer containing 0.15 *M* sodium chloride and 0.01% sodium azide, final pH 7.4.
5. γ-globulin solution: 1.4% bovine γ-globulins (Sigma, St. Louis, MO) in buffer B.
6. Polyethylene glycol/potassium iodide solution: 20% polyethylene glycol 6000 and 6.25% potassium iodide in buffer B.

3. Method

1. An aliquot (10 μL) of the mixture is retained for counting and the rest is applied to a column (1 × 20 cm) of Sephadex G75 resin (*see* **Notes 1** and **2**).
2. Elution is carried out at a flow rate of 20 mL/h, and fractions (0.6 mL) are automatically collected.
3. Aliquots (10 μL) are counted for radioactivity.
4. An elution profile of radioactivity against fraction number (for a typical profile, *see* **Fig. 1**) is plotted.
5. Immunoreactive protein is identified by reaction with specific antiserum to that protein in the following manner (**steps 6–13**) (*see* **Note 3**).
6. Aliquots (10 μL) of fractions making up the different peaks are diluted so that each gives 10,000 counts/min/100 μL.
7. Each diluted aliquot (100 μL) is incubated with the specific antiserum (100 μL) at 4°C for 4 h.
8. Protein bound to antibody and excess antibody are precipitated by the addition to each sample at 4°C of γ-globulin solution (200 μL) and polyethylene glycol/potassium iodide solution (1 mL) (*see* **Note 4**).
9. Each tube is vortexed and is allowed to stand at 4°C for 15 min.
10. Each tube is centrifuged at 4°C at 5000*g* for 30 min.
11. The supernatants are carefully removed by aspiration at a water pump, and the precipitates at the bottom of the tubes are counted for radioactivity.
12. Estimates of the yield of the radioiodination and specific radioactivity of the iodinated protein may then be made (*see* **Note 5**).
13. When the fractions containing radioiodinated protein have been identified, they are split into small aliquots that can be rapidly frozen or freeze-dried for storage (*see* **Note 6**).

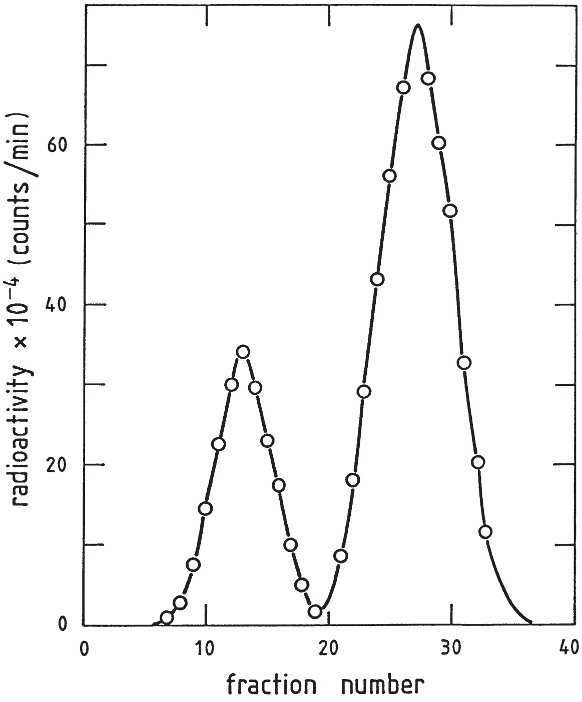

Fig. 1. Gel filtration of radioiodinated kallikrein of rat submandibular gland. The pure enzyme (10 µg) was iodinated with ^{125}I (18.5 MBq) by the chloramine T method. It was then purified on a column (1 × 20 cm) of Sephadex G-75 resin at a flow rate of 20 mL/h and collecting fractions of 0.6 mL. Aliquots (10 µL) of each fraction were measured for radioactivity. By radioimmunoassay, immunoreactive protein was found only in the first peak, and more than 90% of that radioactivity was bound by the antiserum to kallikrein from rat submandibular gland.

4. Notes

1. Gel filtration by high-performance liquid chromatography (HPLC) provides a more rapid and efficient purification of iodinated protein than the conventional liquid chromatography described here, but it does entail the use of expensive columns and apparatus with the attendant danger of their contamination with radioactivity *(2)*.
2. A wide range of gel-filtration resins are available. In choosing a resin, the relative molecular masses (M_r) of the protein and other reactants and products must be borne in mind. Sephadex G-25 resin will separate the labeled protein from the low-mol-wt reagents, such as oxidants and reductants. However, if the labeled protein is contaminated with damaged protein

(e.g., aggregated species), then a gel-filtration resin of higher porosity, such as Sephadex G-100, may produce a more efficient separation of the undamaged, monoiodinated protein.

3. The occurence of immunoreactive protein in more than one peak indicates the presence of polymeric or degradated forms of the iodinated protein. Ideally, iodinated protein should be present in a single, sharp, symmetrical peak. If this is not the case, then it is probably best to repeat the radioiodination under milder conditions or use a different method of iodination.
4. Polyethylene glycol produces precipitation of antibody and antibody-bound protein with little precipitation of unbound protein. Potassium iodide decreases the precipitation by polyethylene glycol of unbound protein ***(3)***.
5. A typical example of the calculation of yield of iodination and specific radioactivity is as follows:

Counts of 10 µL incubation mixture prior to gel filtration = 1,567,925 counts/10 s (1)

Some of the radioiodinated protein is likely to bind to the reaction vessel and other surfaces, so it is best to calculate the radioactivity associated with the labeled protein in terms of the difference between total radioactivity applied to the column and the radioactivity associated with the unreacted iodide.

Counts in 10-µL aliquots of iodide peak = 388,845 counts/10 s (2)

Counts in 10-µL aliquots of protein peak = 1,567,925–388,845 counts/10 s = 1,179,080 counts/10 s (3)

Yield of radioiodination reaction = % incorporation of ^{125}I into protein
(1,179,080 × 100)/1,567,925 = 75.2% (4)

Amount of radioactivity incorporated into protein = % incorporation × original
radioactivity= 75.2% × 18.5 MBq = 13.9 MBq (5)

Specific radioactivity = (amount of radioactivity in protein/amount of protein used)
= (13.9 MBq/10 µg) = (1.4 MBq/µg) (6)

6. Each aliquot should be thawed and used only once. Radioiodinated proteins differ markedly in their stability. Some can be stored for several wk (though it must be borne in mind that the half-life of ^{125}I is about 60 d), whereas others can only be kept for several days. If necessary, the labeled protein can be repurified by gel-filtration or ion-exchange chromatography prior to use.

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Purification of IgG by Precipitation with Sodium Sulfate or Ammonium Sulfate

Mark Page and Robin Thorpe

1. Introduction

Addition of appropriate amounts of salts, such as ammonium or sodium sulfate, causes precipitation of IgG *(1)* from all mammals, and can be used for serum, plasma, ascites fluid, and hybridoma culture supernatant. Although such IgG is usually contaminated with other proteins, the ease of these precipitation procedures coupled with the high yield of IgG has led to their wide use in producing enriched IgG preparations. They are suitable for many immunochemical procedures, e.g., production of immunoaffinity columns, and as a starting point for further purification. It is not suitable however for conjugating with radiolabels, enzymes, or biotin since the contaminating proteins will also be conjugated, thereby reducing the efficiency of the labeling and the quality of the reagent. The precipitated IgG is usually very stable, and such preparations are ideally suited for long-term storage or distribution and exchange between laboratories.

Ammonium sulfate precipitation is the most widely used and adaptable procedure, yielding a 40% pure preparation; sodium sulfate can give a purer preparation for some species, e.g., human and monkey.

2. Materials

2.1. Ammonium Sulfate Precipitation

1. Saturated ammonium sulfate solution: Add excess $(NH_4)_2SO_4$ to distilled water (about 950 g to 1 L), and stir overnight at room temperature. Chill at 4°C, and store at this temperature. This solution (in contact with solid salt) is stored at 4°C.
2. PBS: 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4. Store at 4°C.

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2.2. Sodium Sulfate Precipitation

This requires solid sodium sulfate.

3. Methods

3.1. Ammonium Sulfate Precipitation

1. Prepare saturated ammonium sulfate at least 24 h before the solution is required for fractionation. Store at 4°C.
2. Centrifuge serum or plasma for 20–30 min at 10,000g_{av} at 4°C. Discard the pellet (*see* **Note 1**).
3. Cool the serum or plasma to 4°C, and stir slowly. Add saturated ammonium sulfate solution dropwise to produce 35–45% final saturation (*see* **Note 2**). Alternatively, add solid ammonium sulfate to give the desired saturation (2.7 g of ammonium sulfate/10 mL of fluid = 45% saturation). Stir at 4°C for 1–4 h or overnight.
4. Centrifuge at 2000–4000g_{av} for 15–20 min at 4°C (alternatively for small volumes of 1–5 mL, microfuge for 1–2 min). Discard the supernatant, and drain the pellet (carefully invert the tube over a paper tissue).
5. Dissolve the precipitate in 10–20% of the original volume in PBS or other buffer by careful mixing with a spatula or drawing repeatedly into a wide-gage Pasteur pipet. When fully dispersed, add more buffer to give 25–50% of the original volume and dialyze against the required buffer (e.g., PBS) at 4°C overnight with two to three buffer changes. Alternatively, the precipitate can be stored at 4 or −20°C if not required immediately.

3.2. Sodium Sulfate Precipitation (*see* Note 3)

1. Centrifuge the serum or plasma at 10,000g_{av} for 20–30 min. Discard the pellet, warm the serum to 25°C, and stir.
2. Add solid Na_2SO_4 to produce an 18% w/v solution (i.e., add 1.8 g/10 mL), and stir at 25°C for 30 min to 1 h.
3. Centrifuge at 2000–4000g_{av} for 30 min at 25°C.
4. Discard the supernatant, and drain the pellet. Redissolve in the appropriate buffer as described for ammonium sulfate precipitation (**Subheading 3.1., step 5**).

4. Notes

1. If lipid contamination is excessive in ascites fluids, thereby compromising the salt precipitation, add silicone dioxide powder (15 mg/mL) and centrifuge for 20 min at 2000g_{av} *(2)* before adding the ammonium or sodium salt.
2. The use of 35% ammonium sulfate will produce a pure IgG preparation, but will not precipitate all the IgG present in serum or plasma. Increasing saturation to 45% causes precipitation of nearly all IgG, but this will be contaminated with other proteins, including some albumin. Purification using $(NH_4)_2SO_4$ can be improved by repeating the precipitation, but this

may cause some denaturation. Precipitation with 45% $(NH_4)_2SO_4$ is an ideal starting point for further purification steps, e.g., ion-exchange or affinity chromatography and FPLC purification (*see* Chapters 139 and 140).

3. Sodium sulfate may be used for precipitation of IgG instead of ammonium sulfate. The advantage of the sodium salt is that a purer preparation of IgG can be obtained, but this must be determined experimentally. The disadvantages are that yield may be reduced depending on the IgG characteristics of the starting material, IgG concentration, and composition. Fractionation must be carried out at a precise temperature (usually 25°C), since the solubility of Na_2SO_4 is very temperature dependent. Sodium sulfate is usually employed only for the purification of rabbit or human IgG.

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Purification of IgG Using Caprylic Acid

Mark Page and Robin Thorpe

1. Introduction

Caprylic (octanoic) acid can be used to purify mammalian IgG from serum, plasma, ascites fluid, and hybridoma culture supernatant by precipitation of non-IgG protein *(1)* (*see* **Note 1**). Other methods have been described where caprylic acid has been used to precipitate immunoglobulin depending on the concentration used. The concentration of caprylic acid required to purify IgG varies according to species (*see* **Subheading 3., step 2**). For MAb, it is usually necessary to determine experimentally the quantity required to produce the desired purity/yield. Generally, the product is of low to intermediate purity but this will depend on the starting material. Caprylic acid purified IgG preparations can be used for most immunochemical procedures, such as coating plates for antigen capture assays and preparation of immunoaffinity columns, but would not be suitable for conjugation with radioisotopes, enzymes, and biotin where contaminating proteins will reduce the specific activity.

2. Materials

1. 0.6 *M* sodium acetate buffer, pH 4.6. Adjust pH with 0.6 *M* acetic acid.
2. Caprylic acid (free acid).

3. Methods

1. Centrifuge the serum at 10,000 g_{av} for 20–30 min. Discard the pellet and add twice the volume of 0.06 *M* sodium acetate buffer, pH 4.6.
2. Add caprylic acid dropwise while stirring at room temperature. For each 25 mL of serum, use the following amounts of caprylic acid: human and horse, 1.52 mL; goat, 2.0 mL; rabbit, 2.05 mL; cow, 1.7 mL. Stir for 30 min at room temperature.

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3. Centrifuge at 4000 g_{av} for 20–30 min. Retain the supernatant and discard the pellet. Dialyze against the required buffer (e.g., PBS) at 4°C overnight with two or three buffer changes.

4. Note

1. The method can be used before ammonium sulfate precipitation (*see* Chapter 179) to yield a product of higher purity.

Reference

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Purification of IgG Using DEAE-Sepharose Chromatography

Mark Page and Robin Thorpe

1. Introduction

IgG may be purified from serum by a simple one-step ion-exchange chromatography procedure. The method is widely used and works on the principle that IgG has a higher or more basic isoelectric point than most serum proteins. Therefore, if the pH is kept below the isoelectric point of most antibodies, the immunoglobulins do not bind to an anion exchanger and are separated from the majority of serum proteins bound to the column matrix. The high capacity of anion-exchange columns allows for large-scale purification of IgG from serum. The anion-exchange reactive group, diethylaminoethyl (DEAE) covalently linked to Sepharose (e.g., DEAE Sepharose CL-6B, Pharmacia, Uppsala, Sweden) is useful for this purpose. It is provided preswollen and ready for packing into a column, and is robust and has high binding capacity. Furthermore, it is relatively stable to changes in ionic strength and pH. Other matrices (e.g., DEAE cellulose) are provided as solids, and will therefore require preparation and equilibration ***(1)***.

This procedure does not work well for murine IgG or preparations containing mouse or rat MAb, since these do not generally have the high pI values that IgGs of other species have. Other possible problems are that some immunoglobulins are unstable at low-ionic strength, e.g., mouse IgG_3, and precipitation may occur during the ion-exchange procedure. The product is of high purity (>90%) and can be used for most immunochemical procedures including conjugation with radioisotopes, enzymes, and so on, where pure IgG is required.

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2. Materials

1. DEAE Sepharose CL-6B.
2. 0.07 *M* sodium phosphate buffer, pH 6.3.
3. 1 *M* NaCl.
4. Sodium azide.
5. Chromatography column (*see* **Note 1**).

3. Methods

1. Dialyze the serum (preferably ammonium sulfate fractionated; *see* Chapter 179) against 0.07 *M* sodium phosphate buffer, pH 6.3, exhaustively (at least two changes over a 24-h period) at a ratio of at least 1 vol of sample to 100 vol of buffer.
2. Apply the sample to the column, and wash the ion exchanger with 2 column volumes of sodium phosphate buffer. Collect the wash, which will contain IgG, and monitor the absorbance of the eluate at 280 nm (A_{280}). Stop collecting fractions when the A_{280} falls to baseline.
3. Regenerate the column by passing through 2–3 column volumes of phosphate buffer containing 1 *M* NaCl.
4. Wash thoroughly in phosphate buffer (2–3 column volumes), and store in buffer containing 0.1% NaN_3.
5. Pool the fractions from **step 2** and measure the A_{280} (*see* **Note 2**).

4. Notes

1. The column size will vary according to the user's requirements or the amount of antibody required. Matrix binding capacities are given by manufacturers and should be used as a guide.
2. The extinction coefficient ($E^{1\%}_{280}$) of human IgG is 13.6 (i.e., a 1 mg/mL solution has an A_{280} of 1.36). This can be used as an approximate value for IgGs from other sources.

Reference

1. Johnstone, A. and Thorpe, R. (1996) *Immunochemistry in Practice,* 3rd ed. Blackwell Scientific, Oxford, UK.

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Purification of IgG Using Ion-Exchange HPLC

Carl Dolman, Mark Page, and Robin Thorpe

1. Introduction

Conventional ion-exchange chromatography separates molecules by adsorbing proteins onto the ion-exchange resins that are then selectively eluted by slowly increasing the ionic strength (this disrupts ionic interactions between the protein and column matrix competitively) or by altering the pH (the reactive groups on the proteins lose their charge). Anion-exchange groups (such as diethylaminoethyl; [DEAE]) covalently linked to a support matrix (such as Sepharose) can be used to purify IgG in which the pH of the mobile-phase buffer is raised above the p*I* or IgG, thus allowing most of the antibodies to bind to the DEAE matrix. Compare this method with that described in Chapter 181 in which the IgG passes through the column. The procedure can be carried out using a laboratory-prepared column that is washed and eluted under gravity ***(1)***; however, high-performance liquid chromatography (HPLC) provides improved reproducibility (because the sophisticated pumps and accurate timers), speed (because of the small high-capacity columns), and increased resolution (because of the fine resins and control systems).

2. Materials

1. Anion-exchanger (e.g., Mono-Q HR 5/5 or HR 10/10, Pharmacia, Uppsala, Sweden.
2. Buffer A: 0.02 *M* triethanolamine, pH 7.7.
3. Buffer B: Buffer A containing 1 *M* NaCl.
4. 2 *M* NaOH.
5. Sodium azide.

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3. Methods

1. Prepare serum by ammonium sulfate precipitation (45% saturation; *see* Chapter 179). Redissolve the precipitate in 0.02 *M* triethanolamine buffer, pH 7.7, and dialyze overnight against this buffer at 4°C. Filter the sample (*see* **Note 1**) before use (0.2 µm).
2. Assemble the HPLC system according to the manufacturer's instructions for use with the Mono-Q ion-exchange column.
3. Equilibrate the column with 0.2 *M* triethanolamine buffer, pH 7.7 (buffer A). Run a blank gradient from 0 to 100% buffer B (buffer A + 1 *M* NaCl). Use a flow rate of 4–6 mL/min for this and subsequent steps.
4. Load the sample depending on column size. Refer to the manufacturer's instruction for loading capacities of the columns.
5. Equilibrate the column with buffer A for at least 10 min.
6. Set the sensitivity in the UV monitor control unit, and zero the baseline.
7. Apply a salt gradient from 0 to 28% buffer B for about 30 min (*see* **Note 2**). Follow with 100% 1 *M* NaCl for 15 min to purge the column of remaining proteins.
8. Wash the Mono-Q ion-exchange with at least 3 column volumes each of 2 *M* NaOH followed by 2 *M* NaCl.
9. Store the Mono-Q ion-exchange column in distilled water containing 0.02% NaN_3.

4. Notes

1. It is essential that the sample and all buffers be filtered using 0.2-µm filters. All buffers must be degassed by vacuum pressure.
2. Using Mono-Q, IgG elutes between 10% and 25% buffer B, usually approx 15%. When IgG elutes at 25% (dependent on p*I*), then it will tend to coelute with albumin, which elutes at approx 27%. When this occurs, alternative purification methods should be employed.
3. Other anion-exchange media can be substituted for Mono-Q, for example, Anagel TSK DEAE (Anachem, Luton, UK).

Reference

1. Johnstone, A. and Thorpe, R. (1996) *Immunochemistry in Practice*, 3rd edition, Blackwell Scientific, Oxford, UK.

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Purification of IgG by Precipitation with Polyethylene Glycol (PEG)

Mark Page and Robin Thorpe

1. Introduction

PEG precipitation works well for IgM, but is less efficient for IgG; salt precipitation methods are usually recommended for IgG. PEG precipitation may be preferred in multistep purifications that use ion-exchange columns, because the ionic strength of the Ig is not altered. Furthermore, it is a very mild procedure that usually results in little denaturation of antibody. This procedure is applicable to both polyclonal antisera and most MAb containing fluids.

2. Materials

1. PEG solution: 20% (w/v) PEG 6000 in PBS.
2. PBS: 0.14 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4.

3. Methods

1. Cool a 20% w/v PEG 6000 solution to 4°C.
2. Prepare serum/ascitic fluid, and so forth, for fractionation by centrifugation at $10{,}000g_{av}$ for 20–30 min at 4°C. Discard the pellet. Cool to 4°C.
3. Slowly stir the antibody containing fluid, and add an equal volume of 20% PEG dropwise (*see* **Note 1**). Continue stirring for 20–30 min.
4. Centrifuge at 2000–$4000g_{av}$ for 30 min at 4°C. Discard the supernatant (*see* **Note 2**), and drain the pellet. Resuspend in PBS or other buffer as described for ammonium sulfate precipitation (*see* Chapter 179).

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4. Notes

1. Although the procedure works fairly well for most antibodies, it may produce a fairly heavy contamination with non-IgG proteins. If this is the case, reduce the concentration of PEG in 2% steps until the desired purification is achieved. Therefore, carry out a pilot-scale experiment before fractionating all of the sample.
2. PEG precipitation does not work for some antibodies. If the procedure is to be used for a valuable antibody for the first time, keep the supernatant in case precipitation has been inefficient.

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Purification of IgG Using Protein A or Protein G

Mark Page and Robin Thorpe

1. Introduction

Some strains of *Staphylococcus aureus* synthesize protein A, a group-specific ligand that binds to the Fc region of IgG from many species ***(1,2)***. Protein A does not bind all subclasses of IgG, e.g., human IgG_3, mouse IgG_3, sheep IgG_1, and some subclasses bind only weakly, e.g., mouse IgG_1. For some species, IgG does not bind to protein A at all, e.g., rat, chicken, goat, and some MAbs show abnormal affinity for the protein. These properties make the use of protein A for IgG purification limited in certain cases, although it can be used to an advantage in separating IgG subclasses from mouse serum ***(3)***. Protein G (derived from groups C and G *Streptococci*) also binds to IgG Fc with some differences in species specificity from protein A. Protein G binds to IgG of most species, including rat and goat, and recognizes most subclasses (including human IgG_3 and mouse IgG_1), but has a lower binding capacity. Protein G also has a high affinity for albumin, although recombinant DNA forms now exist in which the albumin-binding site has been spliced out, and are therefore very useful for affinity chromatography. Other streptococcal immunoglobulin binding proteins are protein H (binds IgG Fc), protein B, which binds IgA and protein Arp, which binds IgG & IgA. These are not generally available for immunochemical use.

Another bacterial IgG-binding protein (protein L) has been identified ***(4)***. Derived from *Peptostreptococcus magnus,* it binds to some κ (but not λ) chains. Furthermore, protein L binds to only some light-chain subtypes, although immunoglobulins from many species are recognized ***(5,6)***.

Finally, hybrid molecules produced by recombinant DNA procedures, comprising the appropriate regions of IgG-binding proteins (e.g., protein L/G, protein L/A) also have considerable scope in immunochemical techniques. These proteins are therefore very useful in the purification of IgG by affinity chromatography. Columns are commercially available (MabTrap G II, Pharmacia,

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Uppsala, Sweden) or can be prepared in the laboratory. The product of this method is of high purity and is useful for most immunochemical procedures including affinity chromatography and conjugation with radioisotopes, enzymes, biotin, and so forth.

2. Materials

1. PBS: 0.14 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2 PO_4, 8.1 m*M* Na_2HPO_4.
2. Sodium azide.
3. Dissociating buffer: 0.1 *M* glycine-HCl, pH 3.5. Adjust the pH with 2 *M* HCl.
4. 1 *M* Tris.
5. Binding buffer: Optimal binding performance occurs using a buffer system between pH 7.5 and 8.0. Suggested buffers include 0.1 *M* Tris-HCl, 0.15 *M* NaCl, pH 7.5; 0.05 *M* sodium borate, 0.15 *M* NaCl, pH 8.0; and 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.5.
6. IgG preparation: serum ascitic fluid, or hybridoma culture supernatant.
7. Protein A, G column.

3. Methods

Refer to Chapter 186 for CNBr activation of Sepharose and coupling of protein A, G.

1. Wash the column with an appropriate binding buffer.
2. Pre-elute the column with dissociating buffer, 0.1 *M* glycine-HCl, pH 3.5.
3. Equilibrate the column with binding buffer.
4. Prepare IgG sample: If the preparation is serum, plasma, or ascitic fluid, dilute it at least 1:1 in binding buffer and filter through 0.45-µm filter. Salt-fractionated preparations (*see* Chapter 179) do not require dilution, but the protein concentration should be adjusted to approx 1–5 mg/mL. Hybridoma culture supernatants do not require dilution.
5. Apply sample to column at no more than 10 mg IgG/2-mL column.
6. Wash the column with binding buffer until the absorbance at 280 nm is <0.02.
7. Dissociate the IgG-ligand interaction by eluting with dissociating buffer. Monitor the absorbance at 280 nm, and collect the protein peak. Neutralize immediately with alkali (e.g., 1 *M* Tris, unbuffered).
8. Wash the column with binding buffer until the pH returns to that of the binding buffer. Store the column in buffer containing at least 0.15 *M* NaCl and 0.1% sodium azide.
9. Dialyze the IgG preparation against a suitable buffer (e.g., PBS) to remove glycine/Tris.

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Analysis and Purification of IgG Using Size-Exclusion High Performance Liquid Chromatography (SE-HPLC)

Carl Dolman and Robin Thorpe

1. Introduction

The use of size-exclusion chromatography (SEC; also known as gel filtration chromatography) for purification of IgG has been widely used. SEC with dextran, agarose, and polyacrylamide soft gels was one of the first techniques developed for the purification of proteins. Soft gels, however, cannot tolerate high pressure and require long separation times. They also have limited stability to extremes of pH and to salt. These and other limitations of "conventional" size-exclusion chromatography are responsible for the problems with resolution often observed when analyzing/purifying IgG using such methods. However, many of these problems can be solved by using high performance liquid chromatography (HPLC) systems with appropriate size-exclusion columns.

Silica-based particles such as those used for many HPLC SEC columns are ideal for analysis and purification of IgG, as they are inert, have well-defined pore size, and because of their rigidity can be run at relatively high pressure, allowing reasonable flow rates and relatively quick separation times. Different pore sizes of the silica beads allow fractionation of different ranges of protein molecular weights. The column chosen should therefore have a pore size that allows the fractionation of proteins of molecular weights in the range 10,000–500,000, as this will separate aggregated, dimeric, monomeric, and fragmented immunoglobulins as discrete peaks (e.g., TSK G3000SW; *see* **Fig. 1**). Resolution of peaks is also dependent on column length, and in the past it has been necessary to join two columns to achieve optimal results. This of course doubles the time taken to run a sample. The alternative is to use a column with

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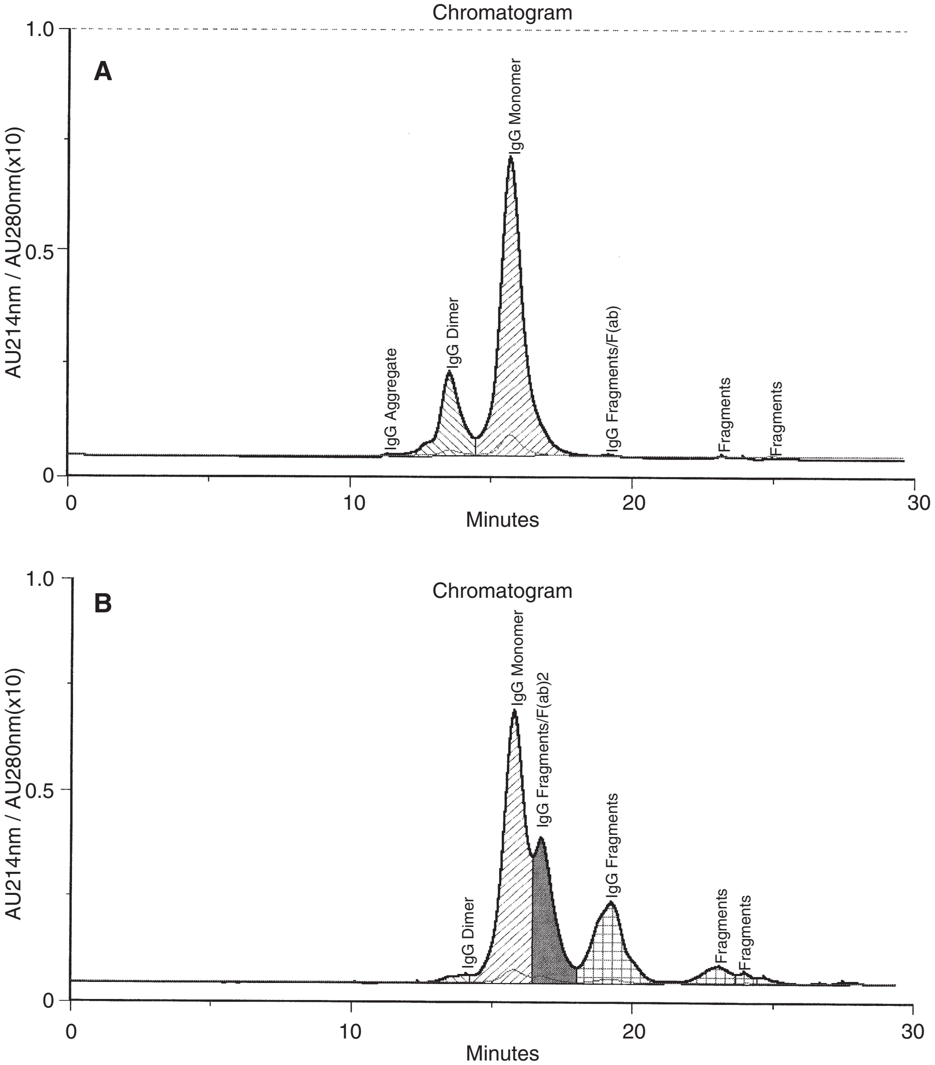

Fig. 1. Typical profiles for intact **(A)** and fragmented **(B)** human therapeutic immunoglobulin products.

smaller bead dimensions, for example, the TSK G3000SW$_{XL}$, which has 5-µm beads compared to the 10-µm beads used for G3000SW columns.

As well as being much quicker than conventional size-exclusion chromatography, SE-HPLC is capable of much increased resolution, and the fully automated systems provide much greater reproducibility than “conventional” chromatography setups.

2. Materials

1. The principal equipment used is a modular HPLC system (e.g., Gilson, Waters) comprising an autosampler, pump, UV-detector, and fraction collector.
2. The equipment is controlled using a Unipoint system controller or equivalent software.
3. TSK G3000SW$_{XL}$ 7.8 mm × 300 mm (or equivalent) column (supplied locally by Anachem, Luton, UK).
4. Mobile phase: 0.2 *M* sodium phosphate, pH 6.0 + 0.1 *M* sodium sulfate, pH 6.0.
5. 18 Mohm deionized water.
6. 50% Methanol in deionized water.

3. Methods (*see* Note 1)

1. Assemble the HPLC components except the column according to the manufacturer's instructions. Equilibrate the system in the mobile phase (*see* **Note 2**).
2. Install the column (and guard column; *see* **Note 3**).
3. Equilibrate the column with mobile phase (*see* **Note 2**), making sure any bacteriostatic storage agent (*see* **Note 4**) is completely removed.
4. Monitor the baseline at 214 nm and/or 280 nm until stable (allow the UV-detector lamp up to 1 h to warm up).
5. Inject sample (*see* **Note 5**).
6. Monitor eluant at 214 nm and/or 280 nm and record data via computer software or integrator.
7. If purifying IgG, set the fraction collector to collect peaks using peak detection or collect 0.1–0.2 mL fractions.

4. Notes

1. All solutions should be 0.22-µm filtered and degassed under vacuum prior to use.
2. The flow rate should be 0.5 mL/min for 7.8 mm internal diameter (i.d.) columns (equal to a linear flow rate of 62.8 cm/h).
3. A guard column can be installed between the autosampler and the main SEC column to prolong the life of the main column, particularly when the sample purity is low.
4. Columns should be stored in the mobile phase with a bacteriostatic agent such as 0.02% azide. Ensure the column is saturated by passing several column volumes of this storage solution through it prior to removing the column from the system.
5. The sample volume is dependent on column size. Volumes above 100 µL will adversely affect sample resolution for 7.8 mm i.d. columns. For analytical purposes use 20 µL of a 1 mg/mL sample. For purification do not exceed a volume of 100 µL or a total load of 1 mg for 7.8 mm × 30 cm columns.

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Purification of IgG Using Affinity Chromatography on Antigen-Ligand Columns

Mark Page and Robin Thorpe

1. Introduction

Affinity chromatography is a particularly powerful procedure, which can be used to purify IgG, subpopulations of IgG, or the antigen binding fraction of IgG present in serum/ascitic fluid/hybridoma culture supernatant. This technique requires the production of a solid matrix to which a ligand having either affinity for the relevant IgG or vice versa has been bound ***(1)***. Examples of ligands useful in this context are:

1. The antigen recognized by the IgG (for isolation of the antigen-specific fraction of the serum/ascitic fluid, and so forth).
2. IgG prepared from an anti-immunoglobulin serum, e.g., rabbit antihuman IgG serum or murine antihuman IgG MAb for the purification of human IgG (*see* **Note 1**).
3. IgG-binding proteins derived from bacteria, e.g., protein A (from *Staphylococcus aureus* Cowan 1 strain) or proteins G or C (from *Streptococcus* and *see* Chapter 184).

The methods for production of such immobilized ligands and for carrying out affinity-purification of IgG are essentially similar, regardless of which ligand is used. Sepharose 4B is probably the most widely used matrix for affinity chromatography, but other materials are available. Activation of Sepharose 4B is usually carried out by reaction with cyanogen bromide (CNBr); this can be carried out in the laboratory before coupling, or ready-activated lyophilized Sepharose can be purchased. The commercial product is obviously more convenient than "homemade" activated Sepharose, but it is more expensive and may be less active.

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2. Materials

1. Sepharose 4B.
2. Sodium carbonate buffer: 0.5 *M* Na_2CO_3, pH 10.5. Adjust pH with 0.1 *M* NaOH.
3. Cyanogen bromide. **(Warning: CNBr is toxic and should be handled in a fume hood.)**
4. Sodium hydroxide: 1 *M*; 4 *M*.
5. Sodium citrate buffer: 0.1 *M* trisodium citrate, pH 6.5. Adjust pH with 0.1 *M* and citric acid.
6. Ligand solution: 2–10 mg/mL in 0.1 *M* sodium citrate buffer, pH 6.5.
7. Ethanolamine buffer: 2 *M* ethanolamine.
8. PBS: 0.14 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4.
9. IgG preparation: serum, ascitic fluid, hybridoma culture supernatant.
10. PBS containing 0.1% sodium azide.
11. Disassociating buffer: 0.1 *M* glycine, pH 2.5. Adjust pH with 1 *M* HCl.
12. 1 *M* Tris-HCl, pH 8.8. Adjust pH with 1 *M* HCl.

3. Method

3.1. Activation of Sepharose with CNBr and Preparation of Immobilized Ligand

Activation of Sepharose with CNBr requires the availability of a fume hood and careful control of the pH of the reaction—failure to do this may lead to the production of dangerous quantities of HCN as well as compromising the quality of the activated Sepharose. CNBr is toxic and volatile. All equipment that has been in contact with CNBr and residual reagents should be soaked in 1 *M* NaOH overnight in a fume hood and washed before discarding/returning to the equipment pool. Manufacturers of ready activated Sepharose provide instructions for coupling (*see* **Note 2**).

1. Wash 10 mL (settled volume) of Sepharose 4B with 1 L of water by vacuum filtration. Resuspend in 18 mL of water (do not allow the Sepharose to dry out).
2. Add 2 mL of 0.5 *M* sodium carbonate buffer, pH 10.5, and stir slowly. Place in a fume hood and immerse the glass pH electrode in the solution.
3. **Carefully** weigh 1.5 g of CNBr into an air-tight container (Note: weigh in a fume hood; wear gloves)—remember to decontaminate equipment that has contacted CNBr in 1 *M* NaOH overnight.
4. Add the CNBr to the stirred Sepharose. Maintain the pH between 10.5 and 11.0 by dropwise addition of 4 *M* NaOH until the pH stabilizes and all the CNBr has dissolved. If the pH rises above 11.5, activation will be inefficient, and the Sepharose should be discarded.
5. Filter the slurry using a sintered glass or Buchner funnel, and wash the Sepharose with 2 L of cold 0.1 *M* sodium citrate buffer, pH 6.5—do not allow the Sepharose to dry out. Carefully discard the filtrate (use care: this contains CNBr).

6. Quickly add the filtered washed Sepharose to the ligand solution (2–10 mg/mL in 0.1 *M* sodium citrate, pH 6.5), and gently mix on a rotator ("windmill") at 4°C overnight (*see* **Note 3**).
7. Add 1 mL of 2 *M* ethanolamine solution, and mix at 4°C for a further 1 h—this blocks unreacted active groups.
8. Pack the Sepharose into a suitable chromatography column (e.g., a syringe barrel fitted with a sintered disk) and wash with 50 mL of PBS. Store at 4°C in PBS containing 0.1% sodium azide.

3.2. Sample Application and Elution

1. Wash the affinity column with PBS. "Pre-elute" with dissociating buffer, e.g., 0.1 *M* glycine-HCl, pH 2.5. Wash with PBS; check that the pH of the eluate is the same as the pH of the PBS (*see* **Note 4**).
2. Apply the sample (filtered through a 0.45-µm membrane) to the column. As a general rule, add an equivalent amount (mole:mole) of IgG in the sample to that of the ligand coupled to the column. Close the column exit, and incubate at room temperature for 15–30 min (*see* **Note 5**).
3. Wash non-IgG material from the column with PBS; monitor the A_{280} as an indicator of protein content.
4. When the A_{280} reaches a low value (approx 0.02), disrupt the ligand–IgG interaction by eluting with dissociating buffer. Monitor the A_{280}, and collect the protein peak into tubes containing 1 *M* Tris-HCl, pH 8.8 (120 µL/1 mL fraction) to neutralize the acidic dissociating buffer.
5. Wash the column with PBS until the eluate is at pH 7.4. Store the column in PBS containing 0.1% azide. Dialyze the IgG preparation against a suitable buffer (e.g., PBS) to remove glycine/Tris.

4. Notes

1. The use of subclass-specific antibodies or MAb allows the immunoaffinity isolation of individual subclasses of IgG.
2. Coupling at pH 6.5 is less efficient than at higher pH, but is less likely to compromise the binding ability of immobilized ligands (especially antibodies).
3. Check the efficiency of coupling by measuring the A_{280} of the ligand before and after coupling. Usually at least 95% of the ligand is bound to the matrix.
4. Elution of bound substances is usually achieved by using a reagent that disrupts noncovalent bonds. These vary from "mild" procedures, such as the use of high salt or high or low pH, to more drastic agents, such as 8 *M* urea, 1% SDS or 5 *M* guanidine hydrochloride. Chaotropic agents, such as 3 *M* thiocyanate or pyrophosphate, may also be used. Usually an eluting agent

is selected that is efficient, but does not appreciably denature the purified molecule; this is often a compromise between the two ideals. In view of this, highly avid polyclonal antisera obtained from hyperimmune animals are often not the best reagents for immunoaffinity purification, as it may be impossible to elute the IgG in a useful form. The 0.1 *M* glycine-HCl buffer, pH 2.5, will elute most IgG, but may denature some MAb. "Pre-elution" of the column with dissociating reagent just before affinity chromatography ensures that the isolated immunoglobulin is minimally contaminated with ligand.

5. The column will only bind to its capacity and therefore some IgG may not be bound; however, this can be saved and passed through the column again. The main problem is with back pressure or even blockage, but this can be reduced by diluting the sample by at least 50% with a suitable buffer (e.g., PBS). Incubation of the IgG containing sample with the ligand matrix is not always necessary, but this will allow maximal binding to occur. Alternatively, slowly recirculate the sample through the column, typically at <0.5 mL/min.

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Purification of IgG Using Thiophilic Chromatography

Mark Page and Robin Thorpe

1. Introduction

Immunoglobulins recognize sulphone groups in close proximity to a thioether group ***(1)***, and, therefore, thiophilic adsorbents provide an additional chromatographic method for the purification of immunoglobulins that can be carried out under mild conditions preserving biological activity. A thiophilic gel is prepared by reducing divinylsulfone (coupled to Sepharose 4B) with β-mercaptoethanol. The product is of intermediate purity and would be useful for further processing, e.g., purification by ion-exchange, size exclusion, and/or affinity chromatography.

2. Materials

1. Sepharose 4B.
2. 0.5 *M* sodium carbonate.
3. Divinylsulfone.
4. Coupling buffer: 0.1 *M* sodium carbonate buffer, pH 9.0.
5. β-mercaptoethanol.
6. Binding buffer: 0.1 *M* Tris-HCl, pH 7.6, containing 0.5 *M* K_2SO_4.
7. IgG preparation: serum, ascitic fluid, or hybridoma culture supernatant.
8. 0.1 *M* ammonium bicarbonate.

3. Methods

Caution: Divinylsulfone is highly toxic and the column preparation procedures should be carried out in a well-ventilated fume cabinet.

1. Wash 100 mL of Sepharose 4B (settled volume) with 1 L of water by vacuum filteration.
2. Resuspend in 100 mL of 0.5 *M* sodium carbonate, and stir slowly.

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3. Add 10 mL of divinylsulfone dropwise over a period of 15 min with constant stirring. After addition is complete, slowly stir the gel suspension for 1 h at room temperature.
4. Wash the activated gel thoroughly with water until the filtrate is no longer acidic (*see* **Note 1**).
5. Wash activated gel with 200 mL of coupling buffer using vacuum filtration and resuspend in 75 mL of coupling buffer.
6. In a well-ventilated fume cabinet, add 10 mL of β-mercaptoethanol to the gel suspension with constant stirring, and continue for 24 h at room temperature (*see* **Note 2**).
7. Filter and wash the gel thoroughly. The gel may be stored at 4°C in 0.02% sodium azide.
8. Pack 4 mL of the gel in a polypropylene column (10 × 1 cm) and equilibrate with 25 mL of binding buffer.
9. Perform chromatography at 4°C. Mix 1 mL of IgG containing sample with 2 mL of binding buffer, and load onto the column.
10. After the sample has entered the gel, wash non-IgG from the column with 20 mL of binding buffer; monitor the A_{280} as an indicator of protein content in the wash until the absorbance returns to background levels.
11. Elute the bound IgG with 0.1 *M* ammonium bicarbonate, and collect into 2-mL fractions. Monitor the protein content by absorbance at 280 nm, and pool the IgG containing fractions (i.e., those with protein absorbance peaks). Dialyze against an appropriate buffer (e.g., PBS) with several changes, and analyze by gel electrophoresis under reducing conditions (*see* Chapter 188).

4. Notes

1. The activated gel can be stored by washing thoroughly in acetone and kept as a suspension in acetone at 4°C.
2. Immobilized ligands prepared by the divinylsulfone method are unstable above pH 8.0.

Reference

1. Porath, J., Maisano, F., and Belew, M. (1985) Thiophilic adsorption—a new method for protein fractionation. *FEBS Lett*. **185,** 306–310.

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Analysis of IgG Fractions by Electrophoresis

Mark Page and Robin Thorpe

1. Introduction (*see* Note 1)

After using a purification procedure it is necessary to obtain some index of purity obtained. One of the simplest methods for assessing purity of an IgG fraction is by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Although "full-size" slab gels can be used with discontinuous buffer systems and stacking gels, the use of a "minigel" procedure, using a Tris-bicine buffer system ***(1)***, rather than the classical Tris-glycine system, is quicker and easier, and gives improved resolution of immunoglobulin light-chains (these are usually smeared with the Tris-glycine system). Gel heights can be restricted to <10 cm and are perfectly adequate for assessing purity and monitoring column fractions.

2. Materials

1. Gel solution: 2.0 mL of 1 *M* Tris, 1 *M* bicine, 4.0 mL 50% w/v acrylamide containing 2.5% w/v *bis*-acrylamide, 0.4 mL 1.5% w/v ammonium persulfate, 0.2 mL 10% w/v SDS.
   Make up to 20 mL with distilled water.
2. Gel running buffer: 2.8 mL 1 *M* Tris, 1 *M* bicine, 1.4 mL 10% w/v SDS.
   Make up to 140 mL with distilled water.
3. Sample buffer: 1.0 g sucrose, 0.2 mL 1 *M* Tris, 1 *M* bicine, 1.0 mL 10% SDS, 0.25 mL 2-mercaptoethanol.
   Make up to 3 mL with distilled water, and add 0.001% w/v Bromophenol blue. Store at –20°C.
4. Coomassie blue R stain: Add coomassie brilliant blue R (0.025 g) to methanol (50 mL), and stir for 10 min. Add distilled water (45 mL) and glacial acetic acid (5 mL). Use within 1 mo.
5. Destain solution: glacial acetic acid (7.5 mL) and methanol (5 mL). Make up to 100 mL with distilled water.

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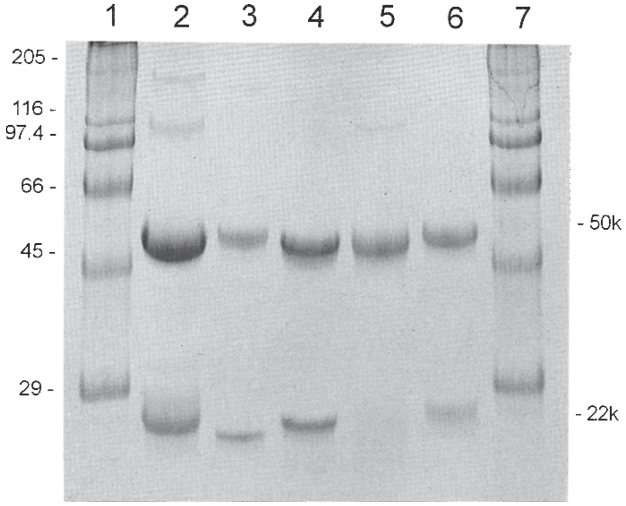

Fig. 1. SDS-PAGE minigel depicting purified IgG preparations derived from human serum (*lane 2*), mouse ascitic flude (*lanes 3* and *4*), rabbit serum (*lane 5*), and sheep serum (*lane 6*). Samples are elctrophoresed under reducing conditions and stained with Coomassie blue. All IgGs consist predominantly of two bands comprising heavy (50,000 M_r) and light (22,000 M_r) chains with no major contaminating proteins. Molecular-weight markers are shown in *lanes 1* and *7* and their molecular weights (in thousands) given on the left.

6. *N,N,N′,N′*-Tetramethylethylenediamine (TEMED).
7. Molecular-weight markers (mol-wt range 200,000–14,000).
8. Purified IgG or column fraction samples.

3. Methods

1. Prepare sample buffer.
2. Adjust antibody preparation to 1 mg/mL in 0.1 *M* Tris, 0.1 *M* bicine (*see* **Note 2**).
3. Mix the sample in the ratio 2:1 with sample buffer.
4. Heat at 100°C for 2–4 min.
5. Prepare gel solution and running buffer as described in **Subheading 2**.
6. Assemble gel mold according to manufacturer's instructions.
7. Add 30 µL TEMED to 10 mL of gel solution, pour this solution between the plates to fill the gap completely, and insert the comb in the top of the mold (there is no stacking gel with this system). Leave for 10 min for gel to polymerize.
8. Remove comb and clamp gel plates into the electrophoresis apparatus. Fill the anode and cathode reservoirs with running buffer.
9. Load the sample(s) (30–50 µL/track), and run an IgG reference standard and/or mol-wt markers in parallel.
10. Electrophorese at 150V for 1.5 h.

11. Remove gel from plates carefully, and stain with Coomassie blue R stain for 2 h (gently rocking) or overnight (stationary) (*see* **Note 3**).
12. Pour off the stain, and rinse briefly in tap water.
13. Add excess destain to the gel. A piece of sponge added during destaining absorbs excess stain. Leave until destaining is complete (usually overnight with gentle agitation).

4. Notes

1. The "minigel" is easily and quickly prepared consisting of a resolving (separating) gel only and takes approx 1.5 h to run once set up. The IgG sample is prepared for electrophoresis under reduced conditions, and is run in parallel with either a reference IgG preparation or with standard mol-wt markers. The heavy chains have a characteristic relative molecular weight of approx 50,000 and the light-chains a molecular weight of 22,000 (**Fig. 1**).
2. The extinction coefficient ($E^{1\%}_{280}$) of human IgG is 13.6 (i.e., a 1 mg/mL solution will have an A_{280} of 1.36).
3. Mark the gel uniquely prior to staining so that its orientation is known. By convention, a small triangle of the bottom left- or right-hand corner of the gel is sliced off.

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Purification of Immunoglobulin Y (IgY) from Chicken Eggs

Christopher R. Bird and Robin Thorpe

1. Introduction

Chickens produce an immunoglobulin G (IgG) homologue, sometimes referred to as IgY (to reflect the differences in the heavy chain domain compared with mammalian IgG), which can be conveniently isolated from the yolk of eggs ***(1)***. The concentration of immunoglobulin in the egg yolk is roughly the same as in serum (10–15 mg/mL), and an average egg can yield approx 80–100 mg of immunoglobulin. Eggs can therefore provide an abundant source of polyclonal antibody that may be aquired noninvasively from eggs laid by immunized chickens ***(2)***. The production of polyclonal antibodies in chickens provides other advantages over using conventional mammalian species. The mechanism of antibody production and organization of the avian immune system is quite different from that of mammals, and as chickens are phylogenetically distant from mammals because of their evolutionary divergence millions of years ago, it is possible to generate antibody responses to highly conserved proteins that do not easily elicit an immune response in mammalian species. Chicken immunoglobulins possess other characteristics that differ from mammalian antibodies that may offer advantages in certain immunological techniques. Most of the interactions via the Fc region in mammalian antibodies do not occur with chicken immunoglobulin. Chicken IgG does not activate mammalian complement systems, and does not react with mammalian rheumatoid factors, and neither protein A or protein G bind to chicken IgG. Although chicken antibodies can be substituted for mammalian antibodies in many techniques (and may even be advantageous) it may be necessary to optimize conditions in certain systems particularly with precipitation techniques where chicken IgG appears to be less efficient then conventional mammalian polyclonal antibodies.

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To isolate chicken immunoglobulins from egg yolk the lipid first has to be removed. This can be achieved by dextran sulfate precipitation after which the chicken immunoglobulins can be purified by sodium sulfate precipitation.

2. Materials

1. Tris-buffered saline (TBS): 0.14 *M* NaCl in 10 m*M* Tris-HCl, pH 7.4.
2. 1 *M* Calcium chloride.
3. Dextran sulphate solution: 10% (w/v) in TBS.
4. Centrifuge.
5. Anhydrous sodium sulfate.
6. Saturated sodium sulfate solution: 36% (w/v) in water.

3. Methods

Break the eggshell and carefully separate the egg yolk from the white to minimize contamination of the yolk with egg white proteins. Place the yolk on a piece of filter paper in which a small hole has been cut. Position the yolk above the hole and pierce the yolk membrane with a needle and collect the yolk in a suitable container (e.g., 50-mL centrifuge tube). The yolk membrane and any remaining egg white will stick to the filter paper. Approximately 10 mL of yolk is obtained from an average sized egg.

1. Dilute the egg yolk with 4 vol of TBS.
2. Centrifuge at 2000–3000*g* for 20 min at room temperature with the brake off.
3. Remove the supernatant, and discard the membrane pellet and add 120 µL of dextran sulfate solution/mL of supernatant, mix well, and incubate at room temperature for 30 min.
4. Add 50 µL of 1 *M* $CaCl_2$/mL and mix well. Incubate for a further 30 min at room temperature.
5. Centrifuge at 2000–3000*g* for 30 min and collect the supernatant, which should be clear; if not, repeat **steps 3** and **4** using half the amounts of dextran sulfate and calcium chloride. The pellet can be washed once at this stage with 50 mL of TBS, and the supernatant combined with the first dextran sulfate supernatant obtained.
6. Adjust the volume of the pooled supernatants to 100 mL with TBS. Stir the supernatant and slowly add 20 g of sodium sulfate until completely dissolved and leave to stand for 30 min at room temperature.
7. Centrifuge for 20 min at 2000–3000*g* and discard the supernatant.
8. Redissolve the precipitate in 10 mL TBS and centrifuge for 20 min at 2000–3000*g*. Collect the supernatant and discard the pellet.
9. Stir the supernatant and slowly add 8 mL of 36% (w/v) sodium sulfate solution and leave to stand for 30 min at room temperature.
10. Centrifuge at 2000–3000*g* for 20 min, discard the supernatant, and redissolve the pellet in 5 mL of TBS. Dialyze and filter the purified immunoglobulin.

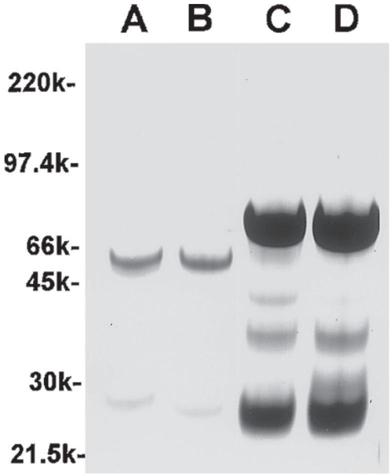

Fig. 1. Coomassie Blue stained SDS-polyacrylamide gel (10% total acrylamide) showing the different mobility of chicken IgG heavy chain compared to mammalian IgG. *Lanes: A*, protein G purified sheep IgG; *B*, therapeutic human IgG; *C* and *D*, two different preparations of purified chicken IgG. All samples were run reduced.

4. Notes

1. Eggs may be stored at 4°C until several have accumulated and then processed together. Alternatively separated egg yolk can be diluted with buffer containing preservative and stored at 4°C prior to purification.
2. 36% (w/v) Sodium sulfate is a supersaturated solution and requires heating to fully dissolve, after which the solution should be stored at 30–40°C before use. The entire procedure for IgG precipitation with sodium sulfate should be carried out at 20–25°C; otherwise the sodium sulfate may precipitate.
3. Purified chicken IgG can sometimes aggregate after freezing and thawing; therefore storing sterile solutions of purified chicken IgG at 4°C may be advisable; alternatively they can be lyophilized for longer term storage.
4. The chicken IgG heavy chain has a different mobility from mammalian IgG, which can be demonstrated by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (**Fig. 1**).

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Affinity Purification of Immunoglobulins Using Protein A Mimetic (PAM)

Giorgio Fassina, Giovanna Palombo, Antonio Verdoliva, and Menotti Ruvo

1. Introduction

While antibodies of the G class can be conveniently purified by affinity chromatography using immobilized protein A or G even at large scale, scaling up purification of IgM, IgA, and IgE and IgY still presents several problems, as specific and cost-effective ligands for these classes of immunoglobulins are not available. Protein A *(1)*, which is widely used for the affinity purification of antibodies from sera or cell culture supernatants, does not recognize immunoglobulins of the M, A, E, and Y classes well and is not used to capture and purify these immunoglobulins from crude sources. Moreover, these two proteins are obtained from microorganisms or genetically modified bacteria, which carries the risk of affecting the safety of the purified antibodies through the presence of contaminants such as viruses, pirogens, or DNA fragments. As a result, the availability of alternative ligands for the affinity purification of antibodies is highly important from an industrial aspect. After immobilization on solid supports, the mannan binding protein (MBP), an affinity ligand for IgM, provides affinity media useful for IgM isolation based on a temperature-dependent interaction of the ligand with the immunoglobulins *(2)*. The use of immobilized MBP for the purification of IgM is based on the adsorption in the presence of calcium at a temperature of 4°C, and the elution at room temperature of adsorbed immunoglobulins in the presence of ethylene-diamineotetraacetic acid (EDTA). This ligand shows low binding affinity for IgG, but binds to bovine and human IgM with lower affinity than murine IgM. However, in addition to the complexity of MBP isolation, functional binding capacities of MBP columns are limited to 1 or 2 mg of IgM per milliliter of support. IgA, which is involved

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in the first specific defense against natural infection *(3)* and represents the second most abundant immunoglobulin in serum *(4)*, can be purified through the combination of different fractionation techniques such as ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration *(5, 6)*. All these procedures are time consuming, labor intensive, and are not compatible with industrial scaling up. Lectin jacalin, isolated from jackfruit seeds *(7)*, binds to IgA and can be conveniently used for the affinity purification of IgA from colostrum or serum *(8)*. However, several aspects limit the use of this lectin for large-scale purification of monoclonal IgA from cell culture supernatants. First, jacalin is a biologically active lectin, being a potent T cell mitogen and a strong B cell polyclonal activator *(9)*, thus requiring a careful control for ligand leakage into the purified preparation. Second, jacalin binds to the carbohydrate moiety of IgA, and D-galactose is required to elute IgA from affinity columns, which is costly and impractical for large-scale operations.

Antibodies of the E class, which represent an extremely important class of immunoglobulins from a biological and clinical point of view, require complex and time-consuming isolation protocols that make their characterization very difficult. The main purification procedure is represented by immunoaffinity chromatography using anti-IgE antibodies immobilized on solid supports *(10, 11)*. Even if selective enough for research application, scaling up immunoaffinity chromatography for preparative applications is very expensive and not easily accomplished. Other approaches for IgE purification include classical chromatographic protocols based on the combination of different sequential procedures such as salting out, affinity chromatography on lysine–Sepharose, ion-exchange, gel filtration, and immuno-affinity chromatography to remove interfering proteins *(12)*. Studies carried out with immobilized protein A show that this protein, known to recognize the immunoglubulin Fc region, does not bind to monoclonal IgE, but binds 12–14% of serum polyclonal IgE. Protein G binds to neither polyclonal nor monoclonal IgE *(13)*.

IgY is an important class of immunoglobulins obtained from chicken egg yolk that represents an economical source of polyclonal antibodies *(14)*. Despite the advantages in production of immunoglobulins from egg yolk in terms of efficiency *(15)*, immunogenicity against mammal proteins *(15)*, and applications in therapeutics and diagnostics *(16, 17)*, only fewer than 2% of the total number of polyclonal antibodies produced worldwide and commercially available are raised in chickens. This low diffusion is related to difficulties in purifying IgY from egg yolk, particularly from the lipidic fraction, which represents the main contaminant. The complex isolation methods described in the literature *(18–20)* and the lack of ligands usable for the affinity purification of IgY make the isolation of this class of immunoglobulins a laborious process that cannot be scaled up easily for industrial applications.

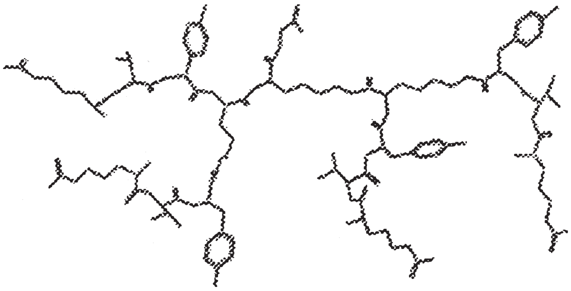

Fig. 1. Structure of PAM (TG19318) (GKK$_2$[YTR]$_4$).

A synthetic peptide ligand (PAM, Protein A Mimetic, TG19318) (*see* **Fig. 1**), derived from the synthesis and the screening of a combinatorial peptide library ***(21)***, has been identified in our laboratory for its ability to recognize, as a protein A mimetic, the constant portion of immunoglobulins. Its applicability in affinity chromatography for the downstream processing of antibodies has been fully established in studies examining the specificity and selectivity for polyclonal and monoclonal antibodies derived from different sources. Ligand specificity is broader than for any existing ligand, as IgGs derived from human, cow, horse, pig, mouse, rat, rabbit, goat, and sheep sera ***(21, 22)***, as well as IgYs derived from egg yolk ***(23)*** have been efficiently purified on PAM affinity columns. The ligand proved useful not only for IgG purification, but also for IgM ***(24)***, IgA ***(25)***, and IgE ***(26)*** isolation from sera or cell culture supernatants (*see* **Table 1**). PAM ligand, a tetrameric tripeptide, can be produced at low cost by means of chemical solution phase or solid phase synthesis in large amounts, does not contain biological contaminants such as viruses, pyrogens, or DNA fragments, as recombinant or extractive ligands such as protein A or G. In addition, the low toxicity of TG19318 and the low molecular weight of the resulting fragments considerably reduce the problems of contamination by leaked ligand, as is the case for protein A. Preliminary experiments suggest that the ligand is more stable to proteolytic digestion when coupled to solid supports, and the enzymatic activity normally found in crude feedstock derived from cell culture supernatants does not lead to noticeable loss of capacity. The ligand can be easily immobilized on preactivated solid supports, as the presence of four peptide chains departing from a central core, of which only a limited number are involved in the coupling with the solid phase, leaves the others fully available for the interaction. All the different supports tested so far maintain the ligand recognition properties for immunoglobulins, even if with different functional capacities. The affinity columns with immobilized PAM are not affected by the presence of denaturants, detergents, or other sanitation reagents commonly used for pirogen removal, and TG19318 columns

Table 1
Immunoglobulins Specificity of PAM (TG 19318)

Ig class	Species	Source
IgG	Human	Serum
	Rabbit	Serum
	Cow	Milk
	Cow	Serum
	Sheep	Serum
	Rat	Serum
(IgG2b)	Rat	Cell supernatant
	Mouse	Serum
(IgG2a)	Mouse	Cell supernatant
(IgG1K)	Mouse	Cell supernatant
	Pig	Serum
	Horse	Serum
	Goat	Serum
IgM	Human	Serum
	Mouse	Serum
	Mouse	Ascites
	Mouse	Cell supernatant
IgA	Human	Serum
	Mouse	Serum
	Mouse	Cell supernatant
IgE	Rat	Ascites
IgY	Chicken	Egg yolk

can withstand a large array of harsh sanitizing agents with no capacity losses. Immunoglobulin adsorption on TG19318 affinity columns occurs with neutral buffers at low ionic strength and at room temperature. Elution of adsorbed immunoglobulins may be achieved under mild conditions not causing immunoglobulin denaturation, simply by changing the buffer pH to acid or alkaline conditions, with acetic acid (pH 3) or sodium bicarbonate (pH 9.0). Increasing the ionic strength of the dissociation buffer favors a more efficient elution of adsorbed antibodies.

Affinity interaction is strong enough to allow purification of antibodies directly from diluted supernatants in which the immunoglobulin concentration is very low, from 10 to 50 µg/mL. The main contaminant, albumin, is always efficiently removed in the purification step with any type of support tested for TG19318 immobilization. Column capacity depends on the type of support used for ligand immobilization, and may range from 10 to 25 mg of immunoglobulin/mL of support.

Purification of antibodies with clinical and therapeutic applications represents a key step in the validation process of these molecules, but the complex and the labor-intensive isolation procedures may alter the quality and the safety of the purified product. In this scenario, the availability of a synthetic ligand able to recognize antibodies independently from their class is of great importance from an industrial point of view, as it may simplify the isolation procedures, leading to lower production costs and reducing considerably the presence of biological contaminants in the purified antibody preparation.

2. Materials

2.1. Synthesis of PAM

1. Automated peptide synthesizer (Perkin-Elmer 431 A).
2. Resin Gly-hydroxymethylphenoxy (Gly-HMP).
3. 9-Fluorenyl-methoxycarbonyl-Lys(9-fluorenyl-methoxycarbonyl) (Fmoc-Lys[Fmoc]).
4. Fmoc-Arg(pentamethylchromane) (Fmoc-Arg[Pmc]).
5. Fmoc-Thr(*O-tert*-butyl) (Fmoc-Thr[OtBu]).
6. Fmoc-Tyr(OtBu).
7. *N*-Methyl-2-pyrrolidone.
8. Piperidine (20% in *N*-methyl-2-pyrrolidone).
9. 1 *M* Dicyclohexylcarbodiimide in *N*-methyl-2-pyrrolidone.
10. 1 *M* Hydroxybenzotriazole (HOBt) dissolved in *N*-methyl-2-pyrrolidone.
11. Methanol.
12. Dichloromethane.
13. Cleavage mixture: Trifluoroacetic acid (TFA)–phenol–water–ethanedithiol–thioanisol.
14. Ether.
15. High-performance liquid chromatography (HPLC) system.
16. Lichrospher RP-8 column (25 × 1 cm internal diameter [i.d.]).
17. Water–acetonitrile–TFA.

2.2. PAM Immobilization on Affinity Media

1. 0.5 *M* NaCl, 0.1 *M* Sodium bicarbonate, pH 8.5.
2. 0.1 *M* Tris-HCl, pH 8.5.
3. 0.1 *M* Acetic acid, 0.5 *M* NaCl, pH 4.0.
4. 0.1 *M* Tris-HCl, 0.5 *M* NaCl, pH 8.0.
5. NHS–Sepharose 4 Fast Flow (Pharmacia Biotech).
6. 3M Emphaze Biosupport Medium AB1 (Pierce).
7. HPLC system.
8. Lichrospher RP-8 column (25 × 1 cm i.d.).
9. Water–acetonitrile–TFA (95:5:0.1).

2.3. Affinity Purification on PAM Columns

1. HPLC/FPLC system.
2. 50–100 m*M* sodium phosphate, pH 7.0.
3. 25–50 m*M* bis-Tris buffer, pH 6.5.

4. 0.1 *M* Acetic acid.
5. 0.1 *M* Sodium bicarbonate, pH 8.5.
6. Protein A–Sepharose 4B (Pharmacia).

3. Methods

3.1. Synthesis of PAM

PAM can be produced in adequate amounts by solid-phase peptide synthesis on automatic peptide synthesizers, such as the Perkin-Elmer model 431A, software version 1.1, according to the synthesis procedure suggested by the manufacturer based on a consolidated methodology well known and widely reported in the literature.

1. Deprotect the Gly-HMP resin (0.1 mmol) by treatment with 3.0 mL of piperidine (20% in *N*-methyl-2-pyrrolidone) for 14 min, at room temperature with stirring.
2. Wash the resin 5× with 2.5 mL of *N*-methyl-2-pyrrolidone for 9 min under agitation at room temperature.
3. In the meantime, preactivate the amino acid residue in position 2 (Fmoc-Lys[Fmoc], 1 mmol) from the C-terminus (1 mmol) by incubation with 1 mL of 1 *M* HOBt dissolved in *N*-methyl-2-pyrrolidone and 1 mL of 1 *M* dicyclohexylcarbodiimide in *N*-methyl-2-pyrrolidone.
4. Incubate the activated amino acid with the resin for 51 min under constant agitation.
5. Wash the resin with *N*-methyl-2-pyrrolidone (four washes for 0.5 min with 2 mL).
6. Subject the resin to a further deprotection cycle with piperidine and a further coupling cycle with the next amino acid.
7. Repeat this sequential step procedure until all the amino acid residues are assembled. In detail, the following amino acid derivatives need to be used: Fmoc-Lys (Fmoc), Fmoc-Arg (Pmc), Fmoc-Thr (OtBu), and Fmoc-Tyr (OtBu).
8. Wash the resin with methanol, dichloromethane, and again with methanol and accurately dry the resin under vacuum for 12 h, after completion of synthesis cycles and removal of the N-terminal Fmoc group by piperidine treatment.
9. Detach protected peptide from resin by incubation of 100 mg of resin with 5 mL of a mixture of trifluoroacetic acid–phenol–water–ethandithiol–thioanisol 84:4:3:3:3 by vol for 2 h at room temperature under agitation.
10. Filter the resin using a sintered glass filter and reduce the filtrate in volume to few milliliters by vacuum evaporation and treat the residual liquid with 50 mL of cold ethyl ether.
11. Separate the precipitated peptidic material by centrifugation and resuspend the centrifuged material in 25 mL of water–acetonitrile–TFA 50:50:0.1, freeze, and lyophilize.
12. Purify the lyophilized material from contaminants by high-performance liquid chromatography (HPLC) using a Lichrospher RP-8 column (25 × 1 cm i.d.), equilibrated at a flow rate of 3 mL/min with water–acetonitrile–TFA 95/5/0.1, and

eluting with a linear gradient of acetonitrile ranging from 5 to 80% in 55 min. Collect material corresponding to the main peak, freeze, and lyophilize.

13. Confirm chemical identity of PAM by determination of:
    a. Amino acid composition;
    b. N-terminal residue;
    c. Molecular weight by mass spectrometry.

3.2. PAM Immobilization on Affinity Media

3.2.1. PAM Immobilization on NHS-Sepharose 4 Fast Flow

1. Dissolve the peptide ligand in 0.1 *M* Na bicarbonate, 0.5 *M* NaCl, pH 8.5.
2. Collect the required amount of NHS-Sepharose 4 Fast Flow, remove the storage solution, and wash with 1 m*M* HCl.
3. Mix the PAM solution with gel suspension in a ratio 2:1 and leave the mixture to incubate for several hours at room temperature under gentle agitation, monitoring the extent of peptide incorporation by reverse phase (RP)-HPLC analysis of reaction mixture at different times (*see* **Note 1**).
4. Wash away excess ligand and incubate with 0.1 *M* Tris, pH 8.5, for 1 h at room temperature to deactivate residual active groups.
5. Wash peptide derivatized resin with at least three cycles of 0.1 *M* acetic acid, 0.5 *M* NaCl, pH 4.0, and 0.1 *M* Tris-HCl, 0.5 *M* NaCl, pH 8.0.
6. Store the product in 20% ethanol at 4°C.

3.2.2. PAM Immobilization on 3M Emphaze Biosupport Medium AB1

1. Dissolve the peptide ligand in 0.2 *M* Na bicarbonate, 0.6 *M* Na citrate, pH 8.0, at a concentration of 5–10 mg/mL.
2. Weigh out the required amount of 3M Emphaze and add the PAM solution directly to the dry beads.
3. Leave the mixture to incubate for several hours at room temperature under gentle agitation, monitoring the extent of peptide incorporation by RP-HPLC analysis of the reaction mixture at different times (*see* **Note 1**).
4. Wash away excess ligand with coupling buffer and incubate with 0.1 *M* Tris, pH 8.5, for 1 h at room temperature to deactivate residual active groups.
5. Wash peptide derivatized resin with at least three cycles of 0.1 *M* acetic acid, 0.5 *M* NaCl, pH 4.0, and 0.1 *M* Tris-HCl, 0.5 *M* NaCl, pH 8.
6. Store the product in 20% ethanol at 4°C.

3.3. Purification of Immunoglobulins on PAM Columns

3.3.1. Affinity Purification of IgG on the PAM-Emphaze Column

1. Dilute sera, ascitic fluids, or cell culture supernatants containing IgG 1:1 (v/v) with the column equilibration buffer, preferably 25–50 m*M bis*-Tris, pH 6.5, filtered through a 0.22-µm filter (Nalgene).
2. Load the sample on to the column equilibrated at a linear flow rate of 60 cm/h with 25–50 m*M bis*-Tris buffer, pH 6.5 (*see* **Notes 2** and **3**), monitoring the effluent

by UV detection at 280 nm. Wash the column with the equilibration buffer until the UV absorbance returns to baseline.

3. Elute bound antibodies with 0.2 *M* acetic acid, pH 3.5 (*see* **Note 4**), wash the column with five volumes of 0.1 *M* acetic acid, and neutralize desorbed material immediately with 1.5 *M* Tris, pH 9.0.
4. Determine by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assay (ELISA) purity and activity. The purity of adsorbed antibodies should be usually very high, ranging from 80% to 95%.
5. Store the column in 20% ethanol or 0.05% sodium azide (w/v) (*see* **Notes 5–7**).

3.3.2. Affinity Purification of IgM on the PAM-Sepharose 4 Fast Flow Column

3.3.2.1. Purification from Cell Culture Supernatants

Immobilized PAM is useful also for the capture of monoclonal IgM directly from crude cell supernatants (*see* **Note 8**) according to the following steps:

1. Load samples of crude cell culture supernatant obtained from stable hybridoma cell lines secreting murine IgM against specific antigens, even if containing a low concentration of IgM (10–100 mg/mL) on a PAM-affinity column equilibrated at a flow rate of 60 cm/h with 50–100 m*M* sodium phosphate, pH 7.0. Samples containing up to 5 mg of IgM may be loaded onto 1-mL bed volume columns. As before, it is recommended to dilute 1:1 (v/v) the samples with the elution buffer prior to application.
2. Wash the column with the equilibration buffer until complete removal of the unretained material is achieved, and then elute with 0.1 *M* acetic acid. Material desorbed by the acid treatment is collected and immediately neutralized.
3. Determine the protein content by the biuret method and IgM content by IgM specific ELISA assay (*see* **Note 9**).

3.3.2.2. Purification from Sera

IgM from sera can be purified by affinity chromatography on a PAM column after a preliminary IgG adsorption step on protein A–Sepharose 4B according to the protocol:

1. Load the serum sample on a protein A–Sepharose 4B affinity column equilibrated with 50 m*M* sodium phosphate, pH 7.0, at a flow rate of 60 cm/h.
2. Collect the column unretained material and dilute 1:1 (v/v) with 100 m*M* sodium phosphate, pH 7.0.
3. Load the protein A unretained fraction on the PAM column equilibrated at a flow rate of 60 cm/h with 50 m*M* sodium phosphate, pH 7.0. Wash the column and elute bound IgM as described previously in **Subheading 3.3.1**.
4. Collect fractions corresponding to the unbound and bound materials for SDS-PAGE analysis and ELISA determination of antibody recovery using an anti-IgM antibody conjugated to peroxidase for detection (*see* **Note 10**).

3.3.3. Affinity Purification of IgA on the PAM-Sepharose Fast Flow Column

3.3.3.1. PURIFICATION FROM CELL CULTURE SUPERNATANTS

Immunoglobulins of the A class secreted in cell culture supernatants derived from the cultivation of hybridoma can be conveniently purified on PAM columns equilibrated with 100 m*M* phosphate buffer, pH 7.0, at a flow rate of 60 cm/h.

1. Dilute sample containing up to 5 mg of IgA 1;1 (v/v) with 100 m*M* sodium phosphate, pH 7.0 and filter through a 0.22-mm filter and then load the sample onto the column.
2. Wash the column with loading buffer until the unbound material is completely removed.
3. Elute the adsorbed immunoglobulins with 0.1 *M* acetic acid and immediately neutralize with 1.5 *M* Tris, pH 9.0. Each fraction is checked for purity by SDS-PAGE and gel filtration analysis (*see* **Note 11**) and for IgA immunoreactivity using an ELISA assay (*see* **Note 12**).

3.3.3.2. PURIFICATION FROM SERA

Isolation of IgA from serum requires the prior removal of the IgG fraction. As in the case of IgM purification from sera, IgA-containing serum needs to be first fractionated on a protein A–Sepharose column, following conventional purification protocols.

1. Dilute 1:1 (v/v) the flow through material from protein A chromatography, lacking IgG and containing mainly IgA, IgM, and albumin, with 100 m*M* sodium phosphate, pH 7.0, and use directly for a subsequent fractionation on PAM columns.
2. Elute the bound fraction, after adsorption and column washing with 100 m*M* sodium phosphate, by a buffer change to 0.1 *M* acetic acid and immediately neutralize.

3.3.4. Affinity Purification of IgE on the PAM-Sepharose 4 Fast Flow Column

Monoclonal IgE obtained from the cultivation of stable hybridoma cell lines or contained in ascitic fluid can also be conveniently purified on PAM affinity columns.

1. Dilute samples containing up to 5 mg of IgE 1:1 with 100 m*M* sodium phosphate, pH 7.0, filter through a 0.22-mm filter, and then directly load onto a PAM column (1 mL bed volume) equilibrated at a flow rate of 1.0 mL/min with 100 m*M* sodium phosphate, pH 7.0, at room temperature.
2. Wash the column after sample loading with loading buffer to remove any unbound material.
3. Elute adsorbed immunoglobulins by a buffer change to 0.1 *M* acetic acid and immediately neutralize with 1.5 *M* Tris, pH 9.0. Each fraction should be checked

for antibody reactivity by ELISA and for purity by SDS-PAGE electrophoresis. As in other cases, no traces of albumin are contaminating the purified IgE preparation. Immunoreactivity of IgE purified on PAM-columns can be determined by ELISA assay on polystyrene microtiter plates (*see* **Note 13**).

3.3.5. Affinity Purification of IgY on the PAM Emphaze Column

Y immunoglobulins from water-soluble yolk extract (*see* **Note 14**) can be isolated in a single-step protocol by affinity chromatography on a PAM column according to the following procedure:

1. Dialyze or dilute the sample 1:1 (v/v) with the starting buffer (25 m*M* *bis*-Tris, pH 6.5), filtered through a 0.45-mm filter (Nalgene).
2. Load the sample onto the column (1 mL bed volume) equilibrated at a flow rate of 1.0 mL/min with 25 m*M* *bis*-Tris, pH 6.5, monitoring the effluent by UV detection at 280 nm.
3. After elution of unbound material, change the eluent to 0.1 *M* acetic acid, pH 3.0, to elute bound material, and neutralize eluted IgY immediately with a few drops of 1 *M* Tris, pH 9.5.
4. Characterize collect fractions by SDS-PAGE, gel permeation, and radial immunodiffusion analysis to determine IgY recovery and purity, and by ELISA, to evaluate the immunoreactivity recovered after purification (*see* **Note 15**).

3.3.6. Sterilization of the PAM Matrix by Autoclaving

PAM resin can be sterilized by autoclaving according to the following procedure:

1. Centrifuge the resin to replace the storage buffer with 0.05 *M* Na phosphate, pH 7.0.
2. Autoclave the sample for up to 30 min at 120°C.
3. Repeat **step 1** to remove autoclaving buffer (*see* **Note 16**).
4. Store the matrix in 20% ethanol at 4°C.

4. Notes

1. PAM immobilization on preactivated solid supports occurs easily, with coupling yields generally between 80 and 95%. Recommended ligand density is between 6 and 10 mg/mL of support.
2. Optimal interaction of immunoglobulins to immobilized PAM occurs in the pH range 6.5–7.5. Compatible buffers are Tris, *bis*-Tris, and sodium phosphate. Phosphate-buffered saline (PBS) is not recommended because the high content of chloride ions interferes with binding. High salt concentrations reduce binding capacity.
3. The use of sodium phosphate as binding buffer, at concentrations from 100 to 200 m*M*, is suggested for samples containing high amounts of phospholipids.

4. Elution of adsorbed immunoglobulins can be performed using acetic acid or 0.1 M sodium bicarbonate, pH 8.5. Addition of sodium chloride to the elution buffer leads to recovery of antibodies in a more concentrated form.
5. PAM column sanitation is easily accomplished, as the ligand is stable to the vast majority of sanitizing agents and is not susceptible of denaturation.
6. Chemical stability of PAM is very high, and in the immobilized form is also sufficiently stable to enzymatic degradation. Columns can be reused for more than 40 purification cycles without appreciable loss of capacity.
7. Removal of adsorbed or precipitated proteins on the columns can be performed by repetitive washings with 0.1 M sodium hydroxide and 1 mM hydrochloridric acid. Check first supports compatibility with these eluents.
8. Binding affinity of PAM is higher for IgM than for IgG. Samples containing both immunoglobulins classes will be enriched in the IgM fractions.
9. Usually very high recovery (80%) is obtained. SDS-PAGE analysis of eluted fractions shows an excellent degree of purification, as no albumin traces are detected in the column-bound fraction, and all the material migrates at the expected molecular weight for IgM. Densitometric scanning of the purified fraction gel lane shows generally purity close to 95%. Column flowthrough material contains, on the other hand, the vast majority of albumin and the other contaminants. Extent of purification can be monitored also by gel filtration chromatography on calibrated columns. Gel filtration profiles of the affinity purified IgM validate SDS-PAGE data, indicating that a single affinity step on PAM columns is sufficient to remove albumin and capture and concentrate the IgM fraction. The effect of purification conditions on the maintenance of antibody antigen binding ability can be evaluated by ELISA assays on microtiter plates coated with the IgM corresponding antigen. For all cases tested, results indicate that the affinity fractionation step is mild and does not lead to loss of immunoreactivity, indicating that the purified antibody is fully active.
10. The vast majority of immunoreactivity (close to 80%) is generally found in the bound fraction, while only little activity is detected in the flow-through fraction. SDS-PAGE analysis indicates that the column bound fraction contains mainly IgM (85% purity), with only trace amounts of IgG or other contaminating proteins. Only IgA are detected as minor contaminants, as this class of immunoglobulins, which is found in sera at very low concentrations, is also recognized by immobilized PAM. Immunoreactivity recovery of IgM from affinity purification can be checked using aliquots of crude material, unbound and bound fractions, directly coated on microtiter plates at the same concentration (10 µg/mL) in 0.1 M sodium carbonate buffer, pH

8.5, overnight at 4°C. After the plates are washed 5× with PBS, wells are then blocked with 100 µL of PBS containing 3% bovine serum albumin (BSA) for 2 h at room temperature, to prevent nonspecific adsorption of proteins. Plates are washed several times with PBS. IgM detection is performed by filling each well with 100 µL of an anti-IgM-peroxidase conjugate solution diluted 1 : 1000 with PBS containing 0.5% BSA, and incubating for 1 h at 37°C. Plates are then washed with PBS 5×, and developed with a chromogenic substrate solution consisting of 0.2 mg/mL ABTS in 0.1 *M* sodium citrate buffer, pH 5.0, containing 5 m*M* hydrogen peroxide. The absorbance at 405 nm of each sample is measured with a Model 2250 EIA Reader (Bio-Rad). If the antigen is available, recovery of immunoreactivity can be evaluated by immobilizing the antigen on microtiter plates, dissolved in 0.1 *M* sodium carbonate buffer, pH 8.5, overnight at 4°C. The plates are washed and saturated as described previously, and filled with crude, unbound and bound materials at the same concentration (10 µg/mL) diluted with PBS–0.5% BSA. The antibody detection and the development of the chromogenic reaction are then carried out as described in **Note 10**.

11. Determination by ELISA of IgA recovery indicates that the column retains 80% of the IgA immunoreactivity initially found in the sample. Gel electrophoretic analysis of the purified fraction indicates the absence of contaminating albumin; however, all the IgM originally present in the sample will be retained by the column. Detection of IgA immunoreactivity in the fractions derived from the affinity step can be accomplished by ELISA by immobilizing IgA containing samples on microtiter plates and detecting IgA with an anti-IgA antibody. SDS-PAGE analysis indicates that the majority of IgA in the sample is retained by the column, and only minute amounts of albumin are detected in the purified preparation. These results are confirmed by the gel filtration analysis, where the column bound fraction shows mainly the presence of IgA. ELISA determination of the IgA content of the column bound and unbound fractions after the purification step indicates that the majority (80–90%) of the initial immunoreactivity is retained by the column.
12. Aliquots of crude material, unbound and bound fractions (100 µL) are incubated on microtiter plates (Falcon 3912) in 0.1 *M* sodium carbonate buffer, pH 8.5, overnight at 4°C. After washing the plates 5× with PBS (50 m*M* phosphate, 150 m*M* sodium chloride), pH 7.5, plate wells are saturated with 100 µL of PBS containing 3% of BSA, for 2 h at room temperature, to prevent nonspecific protein adsorption. Plates are then washed with PBS several times. Detection of IgA antibody is performed by adding to each well 100 µL of an anti-IgA peroxidase conjugate solution (Sigma) diluted 1 : 1000 with PBS–0.5% BSA (PBS-B). The plates are incubated for 1 h

at 37°C, washed with PBS-B containing 0.05% of Tween, then developed with a chromogenic substrate solution consisting of 0.2 mg/mL of ABTS in 0.1 *M* sodium citrate buffer, pH 5.0, containing 5 m*M* hydrogen peroxidase. The absorbance of each sample is measured with a Model 2250 EIA Reader (Bio-Rad).

13. Microtiter plates (Falcon 3912) are incubated with a 10 µg/mL solutions of crude sample, unbound and bound fractions (100 µL/well) in 0.1 *M* sodium carbonate buffer, pH 8.5, overnight at 4°C. After the plates are washed 5× with PBS (50 m*M* phosphate, 150 m*M* sodium chloride), pH 7.5, wells are saturated with 100 µL of a PBS solution containing 3% BSA, for 2 h at room temperature to block the uncoated plastic surface. The wells are then washed with PBS and incubated with the biotinylated antigen (10 µg/mL) in PBS containing 0.5% BSA (PBS-B). After 1 h of incubation the plates are washed 5× with PBS containing 0.05% of Tween (PBS-T), then filled with 100 µL of a streptavidin peroxidase conjugate solution (Sigma) diluted 1 : 1000 with PBS–0.5% BSA. The plates are incubated for 1 h at 37°C, washed with PBS-T 5×, and then developed with a chromogenic substrate solution consisting of 0.2 mg/mL of ABTS in 0.1 *M* sodium citrate buffer, pH 5.0, containing 5 m*M* hydrogen peroxidase. The absorbance at 405 nm of each sample is measured with a Model 2250 EIA Reader (Bio-Rad). For antigen biotinylation, 2 mg of antigen, dissolved in 1 mL of 50 m*M* sodium phosphate buffer, pH 7.5, is treated with 200 µg of biotinamidocaproate *N*-hydroxysuccinimide ester dissolved in 20 µL of dimethyl sulfoxide (DMSO), under agitation at room temperature. After 2 h of incubation, 240 µL of a 1 *M* lysine solution is added to deactivate residual active groups, under stirring for 2 h. At the end the biotinylated antigen is extensively dialyzed against 50 m*M* sodium phosphate, pH 7.5, and used without any further treatment.
14. Water-soluble proteins are separated from the lipidic fraction of egg yolk by the water dilution method. Egg yolk is separated from the white, washed with distilled water to remove as much albumen as possible, diluted 1:9 with acidified distilled water, and incubated at least 6 h at 4°C. After incubation, sample is centrifuged at 10,000*g* for 20 min, the supernatant separated from pellet, filtered on 0.45-µm Nalgene filters, and loaded onto a PAM affinity column. Usually 10.4 mg of antibodies/mL of egg yolk are obtained with a purity close to 30%.
15. Radial immunodiffusion and SDS-PAGE analysis of eluted material show an excellent degree of purification in terms of recovery and purity, both very high and close to 98%. The gel filtration profile of purified IgY validates SDS-PAGE data, indicating that a single affinity step is sufficient to remove all contaminants and capture and concentrate the IgY fraction. The effect of

purification conditions on the maintenance of antibody–antigen recognition can be evaluated by ELISA assays on microtiter plates coated with the IgY corresponding antigen. Results indicate that after the affinity fractionation step, antibodies were recovered fully active, with the majority of the immunoreactivity, about 99%, retrieved in the bound fraction.

16. Chemical and chromatographic stability of PAM resin after sterilization procedure can be tested by monitoring the release of the ligand from the resin by RP-HPLC analysis of the sterilizing buffer and measuring the Ig binding capacity of the matrix in column experiments. The investigations indicate that this treatment leads to significant loss of ligand (up to 5%), so that under process conditions the capacity of PAM columns to bind the immunoglobulins was quite reduced (up to 30%).

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Detection of Serological Cross-Reactions by Western Cross-Blotting

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1. Introduction

Antisera are frequently used tools for the characterization of proteins and peptides because they provide unique information about the structural features of antigens. Proteins that share structural similarities can be identified by serological cross-reactions. The Western blotting technique combines two steps that are characteristic for immunoaffinity chromatography ***(1)***. One is the preparation of monospecific antibodies against a particular antigen and the second is the testing of their reactivity with the same or different antigens from any source of antigenic material.

As a rule, such experiments turn out to be elaborate and time-consuming procedures, consisting of many different steps; particularly because either a purified antigen or a monospecific antibody are basic requirements. In many cases, monospecific antibodies need to be purified from polyspecific antisera by immunoaffinity chromatography. However, only small amounts of monospecific antibody are required for analytical purposes. In such a case it may be sufficient to elute antibodies from selected bands off a Western blot. One protocol, for example, has been published by Beall and Mitchell ***(2)***.

In contrast to experimental setups for the immunochemical analysis of one particular antigen, the method described in this chapter is especially designed for single-step analysis of cross-reactivities of multiple antigens, within the same or between different protein mixtures. The principle is to test antibodies that have bound to particular antigen bands of a Western blot against all antigen bands on a second blot. This is done by electrotransfer of antibodies from one Western blot to a second one, taking advantage of the dissociative effect of chaotropic ions on antigen–antibody complexes.

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The strategy can be dissected into the following steps:

1. Two antigen mixtures are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), each mixture on a separate gel. The samples are loaded onto the entire width of the gels. After electrophoresis, the proteins are blotted onto nitrocellulose (NC) paper.
2. One of the two blots (referred to as the "donor" blot in this text) is incubated with a polyspecific antiserum and then placed onto the second blot ("receptor" blot), upside down, with the protein bands crossing the bands on the second blot. This assembly is wrapped in a dialysis membrane.
3. Antibodies are electrophoretically transferred from the donor blot to the receptor blot in the presence of NaSCN, which dissociates antigen–antibody complexes.
4. The donor blot is then discarded. The receptor blot, still wrapped in the dialysis membrane, is equilibrated with phosphate-buffered saline (PBS) to allow binding of the transferred antibodies to the protein bands.
5. Bound antibodies are detected by use of an enzyme-linked second antibody.

This method may be useful in a wide variety of serological studies. For example, it may help to identify structurally related molecules of different molecular weight within protein mixtures, for example, cellular extracts. It may provide information about the serological relationship of different antigen mixtures, and thereby help to investigate evolutionary distances. It may also find application in analyses of subunit composition of proteins and, in combination with peptide mapping, it can be a useful tool for epitope characterization.

2. Materials

2.1. SDS-PAGE

All solutions for SDS-PAGE should be prepared with chemicals of the highest purity available using double-distilled water.

1. Solution A (acrylamide): 29% (w/v) acrylamide, 1% (w/v) *bis*-acrylamide; store at 4°C, light sensitive, stable for approx 4 wk.
2. Solution B (separating gel buffer): 1.5 *M* Tris-HCl, pH 8.8; store at 4°C.
3. Solution C (stacking gel buffer): 0.5 *M* Tris-HCl, pH 6.8; store at 4°C.
4. SDS: 10% (w/v); store at room temperature.
5. Sample buffer: 62.5 m*M* Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% SDS, 1% (v/v) of a saturated aqueous solution of Bromophenol blue. Stored aliquots of 1 mL at −20°C may be thawed and frozen repeatedly.
6. Electrophoresis buffer: 25 m*M* Tris, 192 m*M* glycine, 0.1% (w/v) SDS. Do not adjust pH.
7. Dithiothreitol (DTT): 1 *M* Dithiotreitol. Stored aliquots of 1 mL at −20°C may be thawed and frozen repeatedly.
8. Iodoacetamide (IAA): 1 *M* IAA. Stored aliquots of 1 mL at −20°C may be thawed and frozen repeatedly.

9. *N*,*N*,*N*′,*N*′-Tetramethylethylendiamine (TEMED): store at 4°C.
10. Ammonium persulfate (APS): 10% (w/v) APS. Store aliquots of 0.1 mL at −20°C.
11. *n*-Butanol (water saturated): Mix equal volumes of *n*-butanol and water and shake well. After separation of the two phases, the upper one is water-saturated butanol. Store at room temperature.

2.2. Electrotransfer

All buffers for this protocol contain methanol. Caution: methanol is toxic and volatile, and should be handled in a fume hood. Wear gloves and protective clothing and do not leave bottles open at the workbench.

1. Buffer A: 0.3 *M* Tris, 20% (v/v) methanol. Do not adjust pH. Store at 4°C.
2. Buffer B: 25 m*M* Tris, 20 % (v/v) methanol. Do not adjust pH. Store at 4°C.
3. Buffer C: 40 m*M* Aminocaproic acid, 25 m*M* Tris, 20% (v/v) methanol. Do not adjust pH. Store at 4°C.

2.3. Cross-Blot

1. Borate-Tween (BT): 50 m*M* $Na_2B_4O_7$, pH 9.3, 0.1 % (v/v) Tween 20. Store at 4°C.
2. SCN-borate-Tween (SBT): 1 *M* NaSCN, 50 m*M* $Na_2B_4O_7$, pH 9.3, 0.1 % (v/v) Tween 20. Store at 4°C.

2.4. Immunostaining

1. PBS: 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 2.7 m*M* KCl, 140 m*M* NaCl. Store at 4°C.
2. PBS-Tween: 0.1% (v/v) Tween 20 in PBS. Store at 4°C.
3. PTS: 8.1 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 2.7 m*M* KCl, 0.5 *M* NaCl, 0.1% (v/v) Tween 20. Store at 4°C.
4. 10 mg/mL Bovine serum albumin (BSA): Store aliquots of 10 mL at −20°C.
5. NC paper.
6. Horseradish peroxidase conjugated antibody with specificity for the immunoglobulin isotype of the test antiserum.
7. Chloronaphthol, solid. Store at −20°C. Caution: irritant to skin, eyes, and respiratory organs. Wear gloves, protective glasses, and clothing, especially when handling the solid substance (buffy crystals). May be purchased as tablets, which can be handled with lower risk.
8. 30% Hydrogen peroxide: Store at 4°C. Caution: strong oxidant. Corrosive. Avoid contact with eyes and skin.

2.5. Miscellaneous

1. Electrophoresis apparatus for SDS-PAGE: Mini-Protean (Bio-Rad, Richmond, CA) or equivalent.
2. Gradient mixer for 2 × 5 mL.
3. Semidry blotting apparatus.

3. Methods

Most conveniently, this protocol may be carried out according to the following time schedule:

Day 1: Casting two SDS gels.
Day 2: Casting the stacking gels.
Running SDS-PAGE.
Blotting of the gels onto NC.
Incubating one blot and of reference strips from both blots with antiserum.
Cross-blotting.
Reequilibration overnight.
Day 3: Incubation with second antibody.
Detection of spots by enzyme reaction.

This schedule proved convenient in our laboratory. Other schedules are possible, as time values for antibody incubations and washing steps given are minimum requirements from our experience and may be different with other materials.

3.1. SDS-PAGE

3.1.1. Casting Gradient Gels

Volumes are given for the Mini-Protean gel system (Bio-Rad) for gels of 83 × 55 × 1.5 mm.

1. Clean glass plates with detergent, rinse thoroughly with tap water followed by double-distilled (dd-) water, and let dry.
2. Assemble glass plates and prepare two gel solutions as follows:

5% Acrylamide:	1.65 mL of solution A; 2.50 mL of solution B; 5.73 mL of dd-water;
20% Acrylamide:	6.65 mL of solution A; 2.50 mL of solution B; 0.73 mL of dd-water.

   Degas under vacuum (e.g., in a sidearm flask) and add 100 µL of SDS.
3. Close the outlet valve and the valve connecting the two chambers of the gradient mixer. Pipet 3.6 mL of the 20% mix into the chamber of the gradient mixer that is connected to the outlet tubing, and then 3.6 mL of the 5% mix into the second chamber. To each chamber, add 1.8 µL of TEMED and 9 µL of APS and mix well.
4. Open the valves and cast the gel at a flow rate of not more than 2.5 mL/min. Control the flow rate either by hydrostatic pressure or by use of a peristaltic pump.
5. Immediately thereafter, rinse the gradient mixer with water and prepare for casting the second gel. Carefully overlay the casted gels with 1 mL of water-saturated butanol and polymerize for at least 3 h or overnight at room temperature. Do not move the gels while still fluid.

3.1.2. Sample Preparation

For gels 1.5 mm thick, approx 10–50 µg of protein in a volume of 20–50 µL/cm of gel width may be loaded. However, the optimal protein concentration may vary with the complexity of the sample.

1. Mix equal volumes of protein solution and sample buffer.
2. Add 1/20 volume of 1 *M* DTT and boil in a water bath for 5 min.
3. Alkylate free sulfhydryl residues on the proteins by adding 1/5 volume of 1 *M* IAA and incubating at 37°C for 30–60 min (*see* **Note 1**).

3.1.3. Casting Stacking Gels

1. Aspirate butanol from the polymerized gel and rinse the gel surface 3× with water. Let gels dry in an inverted position for 5–10 min (*see* **Notes 2** and **3**).
2. Meanwhile, prepare the stacking gel mix as follows:
   Stacking gel solution: 0.65 mL of solution A;
   1.25 mL of solution C;
   5.0 mL of dd-water.
3. Degas as described in **Subheading 3.1.** and add 50 µL of SDS, 50 µL of APS, and 5 µL of TEMED. Mix carefully and apply 1.3 mL onto each gel. Make sure there is 5 mm remaining from the stacking gel surface to the top of the glass plates for sample application. Overlay with water saturated butanol and polymerize for 10 min (*see* **Note 4**).

3.1.4. Running Electrophoresis

1. Assemble electrophoresis apparatus and fill with electrophoresis buffer.
2. Load protein samples onto gels and run at constant current, starting with 75 V for the Bio-Rad Mini-Protean system. Stop electrophoresis when bromophenol blue tracer dye has reached the bottom of the gel (*see* **Note 5**).

3.2. Electrotransfer onto NC

1. For each gel, cut one sheet of NC paper and 15 pieces of Whatman 3MM paper to fit the dimensions of the separating gel (*see* **Note 6**).
2. For each gel, prewet six sheets of the Whatman paper in buffer A, another six sheets in buffer C, and three sheets in buffer B. Prewet the single sheet of NC paper in buffer B.
3. Disassemble the electrophoresis unit, discard the stacking gel, and mount the blot in the following order, making sure that no air bubbles are trapped between individual stacks:
   a. Place the Whatman stack from buffer A onto the anode plate of the semidry blotting apparatus.
   b. Place the Whatman stack from buffer B on top and cover it with the NC sheet.
   c. Place the gel onto the NC paper, then cover it with the Whatman stack from buffer C.
4. Close the apparatus by mounting the cathode plate on top of the assembly and perform electrophoresis at a constant current of 1.4 mA/cm^2 for 2 h.

3.3. Blocking and Incubation of the Blots with Antiserum

All incubations are done on a laboratory shaker, either at room temperature for the time indicated or at 4°C overnight.

1. Disassemble blotting apparatus and incubate the blot, which is intended as the antibody source (i.e., the "donor" blot) in PBS-Tween at room temperature for 30 min. Incubate the second blot ("receptor" blot) in 1% BSA in PBS for 2 h.
2. Incubate the donor blot with antiserum in PBS-Tween with 1 mg/mL of BSA (*see* **Note 7**).
3. From the central area of both blots, cut square-shaped pieces fitted to the dimension of the separating gel. Make asymmetrical marks on edges of the square-shaped NC pieces to help remember the orientation of the antigen bands. Set aside the remaining pieces of the donor blot in PBS-Tween. Incubate the remaining pieces of the receptor blot with antiserum in PBS-Tween with 1 mg/mL of BSA. These margin pieces will serve as reference strips to identify spots on the cross blot.
4. Place donor blot onto a glass filter and wash with 20 mL of PTS in aliquots of approx 3 mL under vacuum using a sidearm flask. Incubate donor blot in PTS for 10 min on a laboratory shaker.
5. Repeat **step 4** twice (*see* **Note 8**).

3.4. Cross Blot

3.4.1. Electrotransfer of Antibodies

1. Prewet one stack of Whatman paper, 1 cm thick and sufficiently sized to cover the donor blot, in BT and a second, equally sized, stack in SBT.
2. Prewet a piece of dialysis membrane (with a pore size of approx 10 kDa) in BT, which is sufficiently sized to wrap the donor blot (i.e., at least twice the area of the donor blot).
3. Assemble the cross-blot as follows (**Fig. 1**):
   a. Place the Whatman stack soaked in BT onto the anode of the semidry blotting apparatus.
   b. Place the dialysis membrane on top, in such a way that one half covers the Whatman stack while the second half rests beside it on the anode plate.
   c. Place the receptor blot, with the protein bands upside, onto the part of the dialysis membrane, which covers the anodal Whatman stack.
   d. Place the donor blot, with the protein bands downward, onto the receptor blot in such a way that the bands on the donor blot are perpendicular to the bands on the receptor blot (*see* **Note 9**).
   e. Cover the donor blot with the dialysis membrane.
   f. Place the Whatman stack soaked with SBT onto the assembly and cover it with the cathodal plate of the semidry apparatus.
4. Perform electrophoresis at a constant current of 3 mA/cm^2 for 90 min (*see* **Note 10**).

3.4.2. Reequilibration of the Receptor Blot

1. Presoak two Whatman stacks of the same size as in **Subheading 3.4.1., step 1** in PTS.
2. **Important note:** During the following step, take extreme care not to slip the receptor blot against dialysis membrane! (*See* **Note 11**.)

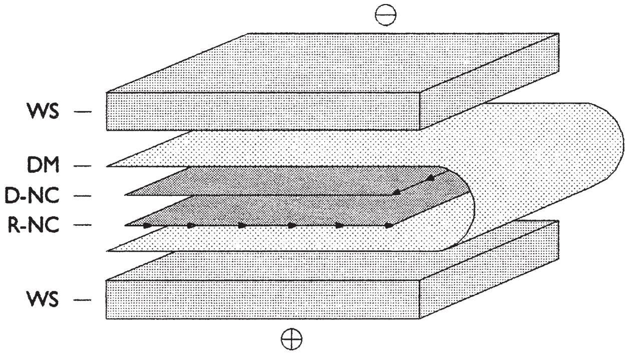

Fig. 1. Experimental setup for a cross-blot experiment: + and – indicate the position of the graphite electrodes of the semidry blotting apparatus. WS, stack of Whatman paper; DM, dialysis membrane; D-NC, donor blot NC sheet; R-NC, receptor blot NC sheet. The perpendicular orientation of the antigens bands on the blots to each other is indicated by arrows. The protein bands on the donor blot are faced downwards against the receptor blot, while the bands on the receptor blot are faced upwards against the donor blot.

- a. Disassemble the semidry apparatus.
- b. Remove the cathodal Whatman stack.
- c. Open the upper part of the dialysis membrane using a pair of forceps.
- d. Remove the donor blot and place into PBS-Tween.
- e. Close the dialysis membrane so that the receptor blot is completely wrapped in it.
- f. Place the receptor blot, wrapped in the dialysis membrane, between two Whatman stacks prewetted in PTS.
- g. Place this assembly into an appropriately sized tray and cover it with a glass plate and a weight of approx 50 g to stabilize it.
- h. Fill tray with additional PTS and incubate overnight without agitation.

3.4.3. Immunostaining

1. Remove the receptor blot from "reequilibration" assembly and wash all NC pieces (i.e., donor blot, receptor blot, and reference strips) in PBS-Tween 3 × 10 min on a laboratory shaker (*see* **Note 12**).
2. Incubate with enzyme-linked second antibody according to the manufacturer's instructions (*see* data sheet) in PBS-Tween with 1 mg/mL of BSA.
3. Wash blots as described in **step 1**.
4. Wash twice for 5 min in PBS.
5. Meanwhile, prepare the staining solution as follows:
   - a. Solubilize approx 10 mg of chloronaphthol in 1–3 mL of ethanol and mix with 50 mL of PBS under vigorous agitation.
   - b. Incubate the solution for 5 min, filter through Whatman 3MM (or equivalent), and add 25 µL of 30% hydrogen peroxide.
6. Incubate NC papers in separate trays in staining solution until bands and spots become visible.
7. Stop the staining reaction by washing the blots in water and dry.

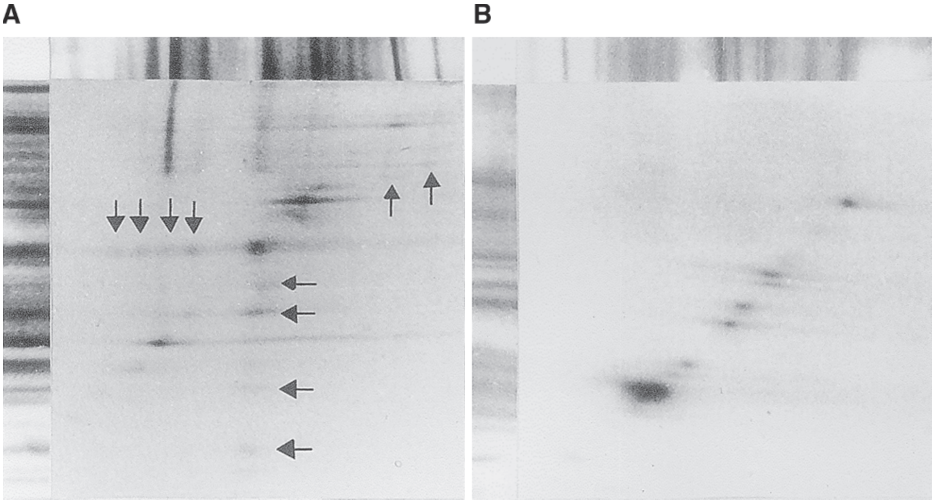

Fig. 2. Two examples of a cross-blot experiment carried out with cytoplasmic extracts of E. coli (taken from ref. 1). To the left of each cross-blot, a reference strip derived from the receptor blot and, on top of each cross-blot, a reference strip derived from the donor blot is included. (**A**) Homologous cross-blot of E. coli B wild-type strain antigens and a rabbit anti E. coli antiserum showing numerous cross-reactions between proteins of different molecular weight (*arrows*). (**B**) Homologous cross-blot of E. coli CSH 57B antigens. The diagonal line of spots indicates a high specificity of the cross-reactions between proteins of the same molecular weight. No cross-reactions between proteins of different molecular weight could be detected in this experiment.

8. For documentation and interpretation of results, place the receptor blot and the reference strips together as shown in **Fig. 2** and photograph (*see* **Note 13**).

4. Notes

1. Samples for SDS-PAGE may be prepared in different ways. Depending on your requirements and intentions, the reduction of inter- and intramolecular disulfide bonds by DTT and alkylation of the resulting sulfhydryl groups may be omitted. However, be aware that remaining APS in the polymerized gel causes an oxidizing environment. This may result in protein oligomerization due to the formation of disulfide bonds between free SH- residues on individual molecules. In many cases, this may be the reason for irreproducible results in SDS-PAGE. For nonreduced samples, it may be beneficial to omit boiling of the sample. In this case, incubate at 37°C for 30 min before applying the sample onto gel.
2. Many types of electrophoresis equipment contain parts made of acrylic glass or equivalent. Avoid butanol or any other organic solvent coming into contact with such parts. This can “corrode” the plastic surface. As a

consequence, the transparency of these parts may be lost with time, which can cause difficulties in handling, (e.g., sample application).

3. While washing the gel surface, do not leave water on the gel for too long a time, as this would dilute the buffer and SDS concentration in the upper part of the gel.
4. The protocol for SDS-PAGE described here uses the discontinuous buffer system of Laemmli and colleagues ***(3)***. For the band sharpening effect of this system a sharp increase in pH and buffer concentration between stacking and separating gel is essential. As soon as the stacking gel is casted, however, the two different buffer systems start to diffuse into each other, and consequently interfere with the beneficial effect of the system. Therefore, it is essential to minimize the time between casting the stacking gel and starting the electrophoresis. For this reason, we do not recommend casting the stacking gels before the samples are ready for application. The polymerization of the stacking gel is complete as soon as a second fluid phase is visible between the gel and the butanol phase.
5. For repeated runs, the electrophoresis buffer may be reused several times. After each run, mix the cathodal and the anodal buffers to restore the pH value. Reuse this buffer only as the anodal buffer in the following runs. For the cathode, use fresh buffer in every run. Reused buffer contains chloride ions from the separating gel of the previous run, which would eliminate the effect of the discontinuous buffer system.
6. Other blotting systems (e.g., in a tank blot module in 10 m*M* Na_2CO_3 or other buffer systems) are also suitable for this purpose. From our experience, the best results are obtained by semidry blotting, especially with respect to homogeneous transfer efficiency.
7. The working dilution of antiserum depends on parameters such as antibody titer and affinity, and therefore varies with the material used. For this reason, no suggestions on antiserum dilution and time of incubation are given in this protocol. It should be adjusted according to the experience of the investigator with the material at hand. However, conditions that ensure a maximum of specificity are a crucial requirement, especially for the cross-blotting method. Therefore, do not use too high a concentration of antiserum and add BSA at 1 mg/mL to minimize unspecific protein–protein interactions. If problems occur, increasing the ionic strength to 0.5 *M* NaCl may be helpful.
8. A rigorous washing procedure was included in the protocol to optimize the washing efficiency. Possibly, this is not necessary for all applications, depending on the materials used. Keep in mind that any antibody, which remains unspecifically attached to the donor blot, will increase background and unspecific signals on the receptor blot.

9. Make sure that the donor and receptor blot are cut of exactly equal size and placed onto each other accurately. This will facilitate the identification of individual spots in the final result. It may be helpful to mark the position of two NC sheets by penetrating them with a needle at three sites after they have been placed together.
10. The relatively long electrophoresis time used in the cross-blot step is intended to maximize the efficiency of antibody elution from the donor blot. However, some antibody species may not survive such a long exposure to 1 *M* NaSCN. This could be indicated by increased unspecific signals or low sensitivity in the final results. In such a case, try a shorter electrophoresis time or a lower concentration of NaSCN in the cathodal Whatman stack. Generally, it may be advantageous to minimize the electrophoresis time in this step so as not to exceed the buffer capacity in the Whatman stacks. This would cause a change in pH, which might affect the direction of antibody migration.
11. This is the most crucial step of the entire procedure. Right after the electrotransfer, the antibodies are not yet bound to the antigens on the receptor blot due to the presence of NaSCN. Instead, they are most likely trapped in the pores of the NC sheet and loosely attached on the surface of the dialysis membrane. Therefore, any movement of the receptor sheet against the anodal surface of the dialysis membrane will result in an extreme decrease of the spot sharpness. The donor blot is removed in this step so that it does not compete with the antigens on the receptor blot during the subsequent reequilibration step. Given a sufficient sensitivity according to our experience with previous experiments, the removal of the donor blot may be omitted.
12 Alternate staining procedures are also possible, for example, second antibody conjugated to alkaline phosphatase and staining with nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP).

   As the staining intensity on the receptor blot is significantly lower than that on the reference strips, one may wish to employ a more sensitive detection system such as luminogenic enzyme substrates. However, some of these systems require the use of special membranes. As of now we have no experience to indicate that these membranes are also compatible with the cross blot procedure.
13. Interpretation of results: Only positive signals should be taken as a result. The failure of a band on the donor blot to react with a band on the receptor blot does not necessarily prove the absence of cross-reactivity. Such negative results could also be due to inefficient elution of an antibody from the donor blot or to denaturation of the eluted antibody. Likewise, the concentration of the antigen and/or antibody on the donor blot may be too low

to give a signal on the receptor blot. As a rule, do not necessarily expect a signal on the cross-blot from donor blot bands which give only faint signals on the donor blot reference strips.

Generally, it is sometimes difficult to judge the specificity of an antigen–antibody reaction. This is more likely for antibodies, which have been exposed to low pH values or chaotropic agents. With cross-blot results, be doubtful about donor blot bands, which give a signal with each band on the receptor blot. Also, receptor blot bands that react with each donor blot band should generally not be taken too seriously, unless special circumstances let you expect such a behavior.

When working with different antigen mixtures on donor and receptor blots, respectively, include a homologous cross-blot as a control and reference. This is done by cross-blotting antibodies from a donor blot to a receptor blot containing the same antigens as the donor blot. Such homologous cross-blots have a "natural" internal reference because each antigen band on the donor blot is crossing "itself" on the receptor blot. In this way, the cross-blot yields a diagonal line of spots that are helpful in estimating both the sensitivity and the specificity you can expect with a particular band on the donor blot. Similarly, a homologous cross-blot may also be carried out with the receptor antigens. This will help to estimate the resistance of the receptor antigens against exposure to the cross-blot conditions.

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Enzymatic Digestion of Monoclonal Antibodies

Sarah M. Andrew

1. Introduction

Originally, digestion of antibodies by proteolytic enzymes was used to study their structure. Many diverse structures can be obtained by fragmentation of the different classes of antibody with different enzymes, or by using the same enzyme and changing the conditions (**Fig. 1**). Not all the fragments obtained have significant binding activity; for example, in several studies by this author, Fv fragments obtained by digestion have been found to have lost their binding activity. Fragmentation of antibody is now usually carried out to introduce required properties (e.g., a decrease in molecular size), or to remove undesirable properties (e.g., nonspecific Fc receptor binding).

The most usual digestions carried out are:

1. Production of bivalent $F(ab')_2$ from mouse McAb IgG;
2. Production of univalent Fab from mouse McAb IgG;
3. Production of bivalent IgMs from mouse McAb IgM; and
4. Production of bivalent $F(ab')_{2\mu}$ from mouse McAb IgM.

$F(ab')_2$ and $F(ab')_{2\mu}$ are produced by digestion with pepsin and Fab is produced by digestion with papain. One useful fragmentation uses papain that has been preactivated with cysteine. This cleaves IgG_1 to produce $F(ab')_2$, and IgG_{2a} IgG_{2b} to produce Fab. It is a very stable fragmentation in which the times of incubation are not at all critical. The IgG-like subunit of IgM (IgMs) is the product of a mild reduction; this is most conveniently done using cysteine, which reduces the IgM and alkylates the subunit, thus preventing reassociation.

After digestion of the antibodies, it is necessary to purify the fragments for two reasons: to separate the fragment from any remaining intact antibody; and to separate the fragments of interest from other miscellaneous fragments produced by the process of digestion. Purification of IgG fragments by protein A affinity

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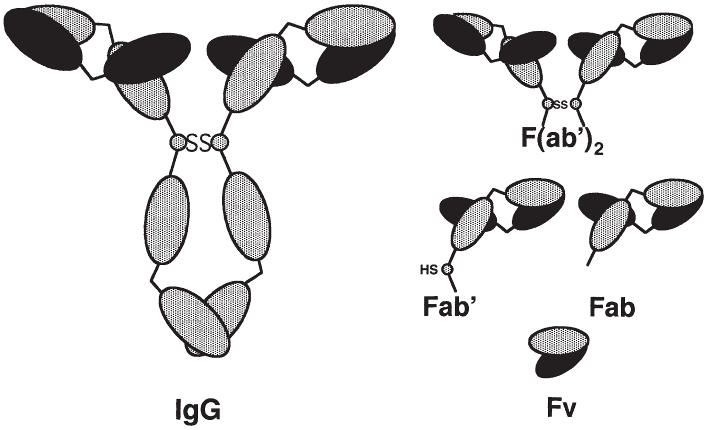

Fig. 1. Diagramatic representation of the major fragments of an IgG molecule that can be produced by enzymatic digestion, as described in the text.

chromatography is possible if an intact Fc region remains after fragmentation, as it does in the case of undigested antibody and, sometimes, in digestions with pepsin. In general this method of purification is rough and ready and size exclusion chromatography is recommended as an additional step.

2. Materials

2.1. Digestion with Pepsin

1. Purified solution of mouse McAb (IgG or IgM) at a concentration of ≥1 mg/mL.
2. 0.2 *M* Sodium acetate buffer brought to pH 4.0 with glacial acetic acid.
3. 0.2 *M* Sodium acetate buffer brought to pH 4.5 with glacial acetic acid.
4. 0.1 mg/mL pepsin (Sigma, St. Louis, MO) in acetate buffer at appropriate pH (*see* **Subheadings 3.1.** and **3.2.**).
5. 2 *M* Tris base.
6. Phosphate-buffered saline (PBS) brought to pH 8.0 using NaOH.
7. Protein A-Sepharose CL-4B (Sigma number P 3391) swollen in PBS, pH 8.0, and packed into a 10 × 100 mm column, or a 1-mL HiTrap Protein A column (Sigma).
8. PBS.
9. A size exclusion column equivalent to 26 × 900 mm Sephacryl S-200 (Pharmacia, Uppsala, Sweden).
10. Centriprep 100 concentrator (Amicon, Beverly MA; Stonehouse, Glocestershire, UK).

2.2. Digestion with Papain

1. Purified solution of mouse IgG McAb at a concentration of ≥1 mg/mL.
2. PBS made to 0.02 *M* with respect to ethylenediaminetetra-acetic acid (EDTA) and 0.2 *M* with respect to cysteine.

3. Iodoacetamide crystals.
4. PBS brought to pH 8.0 using NaOH.
5. Protein A-Sepharose CL-4B (Sigma number) swollen in PBS, pH 8.0, and packed into 10 × 100 mm column, or a 1 mL HiTrap Protein A column (Sigma).
6. PBS.
7. A size exclusion column equivalent to 26 × 900 mm Sephacryl S-200 (Pharmacia) (optional).

2.3. Reduction and Alkylation with Cysteine

1. Purified solution of mouse IgM McAb at a concentration of ≥1 mg/mL in PBS.
2. 0.1 *M* cysteine stock solution in PBS (L-cysteine free base, Sigma).
3. Borate-buffered saline: 0.015 *M* sodium borate, 0.15 *M* sodium chloride, made to pH 8.5 with Sodium hydroxide.
4. A size exclusion column equivalent to 26 × 900 mm Sephacryl S-200 (Pharmacia).

3. Methods

3.1. Preparation of $F(ab')_2$ from IgG

The various subclasses of mouse IgG vary in their susceptibility to pepsin fragmentation. IgG_1 is quite resistant to digestion and and it is impossible to fragment IgG_{2b} to $F(ab')_2$ because it breaks down to Fab (*see* **Notes 1–3**). The method below is one that should work in most cases.

1. Dialyze the IgG against acetate buffer, pH 4.0, overnight at 4°C. Use any known amount of antibody between 1 and 20 mg.
2. Determine the concentration at A_{280}.
3. Add 0.1 mg/mL pepsin in acetate buffer, pH 4.0, to give an enzyme-to-antibody ratio of 1:20 (w:w).
4. Incubate for 6 h in a water bath at 37°C.
5. Stop the reaction by adding sufficient Tris base to bring the pH to roughly 8.0 (start by adding 50 µL Tris, mix, and test the pH with pH paper).
6. Dialyze the mixture against PBS, pH 8.0, overnight.
7. Equilibrate the protein A column with PBS, pH 8.0, and load the dialyzed mixture onto it 1 mL at a time. Collect the unbound fraction that contains the $F(ab')_2$ fragments.
8. If further purity is desired, the mixture should be concentrated to a volume of ≤3 mL and added to a precalibrated size exclusion column (26 × 900 mm Sephacryl S-200 or equivalent). At this stage regular PBS can be used to equilibrate the column and elute the fractions.
9. Collect 2.5-mL fractions over the molecular weight range of $F(ab')_2$ (110 kDa). The purity of the product can be assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in nonreducing conditions and in reducing conditions when a doublet at 25 kDa is seen.

3.2. Preparation of F(ab′)$_{2\mu}$ from IgM (*see* **Note 3**)

1. Dialyze the IgM against acetate buffer, pH 4.5, overnight at 4°C.
2. Determine the concentration at A_{280} (*see* Chapter 1).
3. Add 0.1 mg/mL pepsin in acetate buffer, pH 4.5, to give an enzyme-to-antibody ratio of 1:20 (w:w).
4. Incubate for between 6 and 12 h in a water bath at 37°C.
5. Stop the reaction by adding sufficient Tris base to bring the pH to roughly 8.0 (start by adding 50 µL Tris, mix, and test the pH with pH paper).
6. Dialyze the mixture against PBS overnight, or concentrate and change the buffer using a Centriprep 100 concentrator.
7. Concentrate the mixture to a volume of ≤3 mL and add to a precalibrated size exclusion column (26 × 900 mm Sephacryl S-200 or equivalent). Elute fractions of between 1 and 2.5 mL. The molecular weight of F(ab′)$_{2\mu}$ is 130 kDa. Assess purity on an 8% polyacrylamide gel under nonreducing conditions.

3.3. Preparation of Fab from IgG

Again, there is a variable susceptibility to the enzyme between the IgG subclasses. The method described is one that should give good results in most cases. If fragments are not obtained, variations in the concentration of enzyme or time of incubation can be tried. *See* **Notes 1**, **2**, and **4** for further information.

1. Use IgG in PBS at a concentration between 1 and 5 mg/mL.
2. Dissolve sufficient papain in an equal volume of digestion buffer to the volume of antibody solution to give a papain-to-antibody ratio of 1:20 (w:w).
3. Add the two equal solutions, one containing antibody and one containing papain together; mix thoroughly but gently.
4. Incubate for 4–6 h at 37°C.
5. Stop the reaction by adding crystalline iodoacetamide to make the mixture 0.03 *M* with respect to iodoacetamide, and dissolve by mixing gently.
6. Dialyze the mixture against PBS, pH 8.0, overnight at 4°C.
7. Equilibrate the protein A column in PBS, pH 8.0, then add the dialyzed mixture to it. Collect the unbound fraction. Wash the column with PBS, pH 8.0, to completely recover the Fab fragments.
8. Concentrate the Fab fragment mixture to a volume <5 mL.
9. Load the mixture containing the Fab fragments onto the size exclusion column equilibrated in PBS. Collect fractions corresponding to a molecular weight of 50 kDa.
10. Check the purity of the final product on a 10% SDS-polyacrylamide gel in nonreducing conditions.

3.4. Preparation of IgG Fragments Using Preactivated Papain

Bivalent F(ab′)$_2$ fragments can be obtained from IgG$_1$ by this method. The protocol is also useful for producing monovalent Fab fragments from IgG$_{2a}$ and IgG$_{2b}$. *See* **Note 4** for further information.

3.4.1. Preactivate the Papain

1. Make up a 2 mg/mL solution of papain in PBS with 0.05 *M* cysteine. Warm at 37°C for 30 min.
2. Equilibrate a PD10 column with acetate/EDTA buffer and apply the papain mixture.
3. Collect 10 × 1 mL fractions eluting with acetate/EDTA buffer. Assay the fractions at A_{280} and pool the two or three fractions containing protein.
4. Calculate the concentration of the preactivated papain using the following formula:

$$A_{280}/2.5 = \text{mg preactivated papain/mL} \qquad (1)$$

3.4.2. Digest the IgG

1. Dialyse the IgG against acetate/EDTA buffer and determine the concentration after dialysis.
2. Mix the preactivated papain solution and the IgG solution in an enzyme-to-antibody ratio of 1:20.
3. Incubate for 6–18 h at 37°C.
4. Stop the reaction by adding crystalline iodoacetamide to a concentration of 0.03 *M*.
5. Dialyse the mixture against PBS, pH 8.0.
6. Purify the fragments as in **Subheading 3.3**.

3.5. Preparation of IgM Subunits from Pentameric IgM **(*see* Note 5)**

1. Add cysteine to IgM in PBS (up to 10 mg in 5 mL); make the solution 0.05 *M* with respect to cysteine.
2. Incubate the mixture for 2 h at 37°C.
3. Separate the fragments from intact antibody on a 26 × 900 mm size exclusion column or equivalent. The buffer in the column should be made 3 m*M* with respect to EDTA, if possible, because this prevents reassociation of the IgM fragments. Collect the fractions corresponding to a molecular weight of 180 kDa.

4. Notes

1. It is possible, but expensive, to buy kits for digestion of mouse antibody (Pierce, Warrington, UK). These kits work extremely well and can be a time-saving option. Each laboratory must consider whether the time saved in preparation is worth the extra expenditure on a kit. Fragmentation of antibodies from human and rabbit is described in ***ref. 6***; generally the methods and enzymes used are similar.
2. The most common problems likely to occur when following these methods are that the antibody does not digest or that the molecule overdigests

to produce small unrecognizable fragments. These can be overcome by varying the concentrations of enzyme, the times of digestion and, in the case of pepsin, the pH of the mixture ***(1)***. Generally, it is unwise to embark on fragmentation of IgG if the subclass of the antibody is not known. In digestion with both pepsin and papain the susceptibility to digestion varies with subclass. The order of susceptibility has been found to be $IgG_{2b} > IgG_3 > IgG_{2a} > IgG_1$ ***(2,3)***. Not all antibodies fall into this order (IgG_{2a} can be extremely sensitive to the action of papain in the presence of cysteine) and individual exceptions must be expected.

3. Digestion with pepsin has a great subclass variability. IgG_{2b} does not digest to $F(ab')_2$ fragments at all; the monovalent Fab/c (a single binding site and an intact Fc portion) is produced instead. This molecule has a very similar molecular weight to $F(ab')_2$; thus, is easy to imagine success with the fragmentation. The reason for the problem is thought to be an asymmetric glycosylation of the heavy chains in the molecule. All IgG subclasses can be further digested by pepsin to produce monovalent Fab fragments because there is a site of secondary cleavage on the NH_2-terminal side of the disulfide bonds. Further digestion with pepsin at a pH of 3.5 can produce Fv fragments after approx 3 h of incubation. There are reports of these having activity as antigen-binding fragments ***(4)***, but the personal experience of this author and colleagues suggests this is rare. It is also unfortunately true that it is difficult to produce active fragments from IgM. It has been suggested that IgM heavy chains can be truncated ***(5)***, but this has not been confirmed. The method given for IgM $F(ab')_{2\mu}$ will work, but one should not be too disappointed if the affinity is low.
4. On the whole, papain fragmentations work well and the timings of the incubations are not critical. Initially, care should be taken to mix the papain as it is in suspension; it will dissolve completely at the concentration given in the method. The methods using preactivated papain work extremely well and the incubation times are not at all critical.
5. The digestion to IgMs from IgM causes dissociation of the inter subunit disulfide bonds. It is possible to reduce the intrachain disulfide bonds on further reduction. This is why cysteine is the reducing agent chosen. Reduction by dithiothreitol or mercaptoethanol can be used, but more care is required with the incubations and a separate alkylation step is required.

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How to Make Bispecific Antibodies

Ruth R. French

1. Introduction

This protocol describes the production of bispecific $F(ab')_2$ antibody derivatives (BsAbs) by the linking of two FabЭ fragments via their hinge region SH groups using the bifunctional crosslinker *o*-phenylenedimaleimide (*o*-PDM) as described by Glennie et al. ***(1, 2)***. The procedure is illustrated in **Fig. 1**. The first step is to obtain $F(ab')_2$ from the two parent IgG antibodies. Methods for digestion of IgG to $F(ab')_2$ are described in Chapter 151. Fab′ fragments are then prepared from the two $F(ab')_2$ species by reduction with thiol, thus exposing free SH groups at the hinge region (three SH-groups for mouse IgG1 and IgG2a antibodies) (*see* **Note 1**). One of the FabЭ species (Fab′-A) is selected for alkylation with *o*-PDM. Because *o*-PDM has a strong tendency to crosslink adjacent intramolecular SH-groups, two of the three hinge SH-groups will probably be linked together, leaving a single reactive maleimide group available for conjugation (**Fig. 1**; *see* **Note 2**). Excess *o*-PDM is then removed by column chromatography, and the Fab′-A(mal) is mixed with the second reduced Fab′ (Fab′-B) under conditions favoring the crosslinking of the maleimide and SH groups. When equal amounts of the two parent Fab′ species are used, the major product is bispecific $F(ab')_2$, resulting from the reaction of one Fab′-A(mal) with one of the SH groups at the hinge of Fab′-B. Increasing the proportion of Fab′-A(mal) in the reaction mixture results in a significant amount of $F(ab')_3$ product by the reaction of two molecules of Fab′-A(mal) with two free SH-groups at the hinge of a single Fab′-B molecule (*see* **Note 3**). The remaining free SH groups on Fab′-B are alkylated, and the $F(ab')_2$ bispecific antibody product (Fab′-A × Fab′-B) is separated by gel filtration chromatography. Each stage of the procedure is checked by HPLC.

Using this method, well-defined derivatives are produced with good yield, and the products are easily isolated; starting with 10 mg each of two parent

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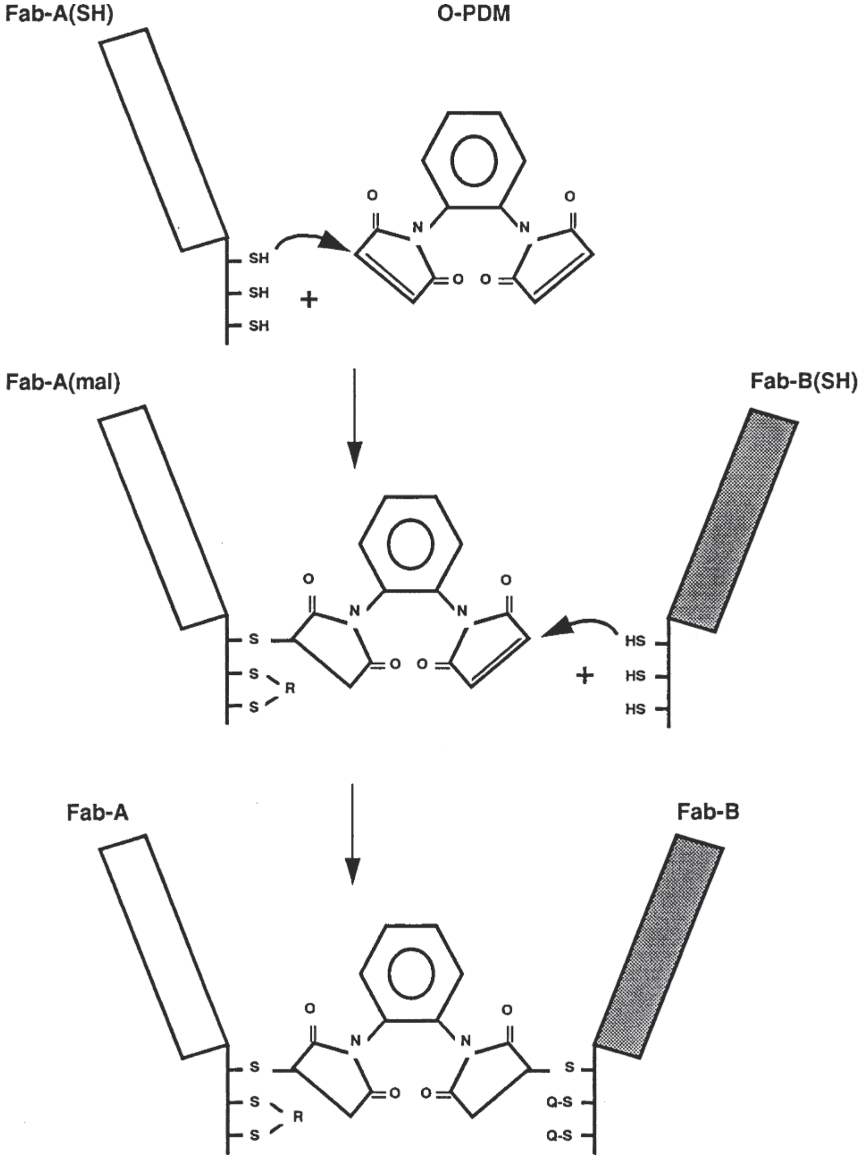

Fig. 1. Preparation of BsAb using *o*-PDM as crosslinker. The F(ab′)$_2$ BsAb illustrated is produced from Fab′ fragments derived from mouse IgG1 or IgG2a antibody. Two adjacent hinge SH-groups of Fab′-A are cross-linked by *o*-PDM (R, *o*-phenylenedisuccinimidyl linkage), leaving one with a free maleimide group for cross-linking with an SH-group at the hinge of Fab′-B. Unconjugated SH-groups at the Fab′-B hinge are blocked by alkylation (Q, carboxyamidomethyl). Increasing the ratio of Fab′-A(mal) to Fab′-B(SH) will favor the production of bispecific F(ab′)$_3$, in which two molecules of Fab′-A(mal) are linked to one molecule of Fab′-B.

F(ab′)$_2$ species, expect to obtain 5–10 mg BsAb. The derivatives can be produced at relatively low cost, and quickly. It is possible to obtain the BsAb product from the parent IgG in five working days. The protocols can be scaled up to produce larger amounts (100–150 mg) of derivative if required. While the derivatives obtained are unlikely to be used for clinical applications because of the problem of immunogenicity, they can provide a very useful tool for proof-of-principle studies in in vitro and in vivo models.

Two potential problems should be taken into consideration when using this technique. First, that the derivatives produced are almost always contaminated with trace amounts of parent IgG antibody or Fc fragments, which are coharvested with the parent F(ab′)$_2$ and the final product. If the presence of Fcthis is likely to be a problem for the application of the BsAb, preparations can be checked for Fc by SDS-PAGE, or assayed tested for Fc by enzyme-linked immunosorbent assay (ELISA), and, if necessary, Fc removed by immunoaffinity chromatography ***(2)***. Second, and probably more important, is the problem of the contamination of the BsAb product with endotoxins, which are lipopolysaccharides (LPS) derived from cell membrane of Gram-negative bacteria. In vivo exposure to LPS, even at low concentrations, can cause a systemic inflammatory reaction, leading to multiple adverse effects, including a pyrogenic reaction and endotoxin shock. LPS acts through activation of the immune system, with the release of a range of pro-inflammatory mediators, such as tumor necrosis factor, IL-6 and IL-1, and consequently LPS contamination can complicate the study of the biological effects of BsAb, both in vitro or in vivo. During the preparation of the parent F(ab′)$_2$ and the BsAb, steps should be taken to avoid endotoxin contamination; products for the the measurement and removal of endotoxins are included in **Note 9**.

2. Materials

2.1. Reagents

To avoid endotoxin contamination of products, all reagents are prepared using commercially available endotoxin-free water, and glassware is cleaned using 0.2 M NaOH followed by thorough rinsing with endotoxin-free water.

1. 2 *M* TE8: 2 *M* Tris-HCl, pH 8.0, 100 m*M* ethylenediaminetetra-acetic acid (EDTA). Prepare 0.2 *M* TE8 from 2 *M* stock.
2. F(ab′)$_2$ reducing solution: 220 m*M* 2-ME, 1 m*M* EDTA. Make up 10 mL. Use a fume cupboard.
3. Sephadex G25 (Pharmacia, Uppsala, Sweden) and Ultragel AcA44 (Biosepra S. A., Villeneuve la Garenne, France) gel filtration media.
4. G25 column buffer (50 m*M* AE); 3.35 g sodium acetate, 526 µL glacial acetic acid, 0.186 g EDTA, made up to 1 L. Degas before use under vacuum or using nitrogen.
5. High performance liquid chromatography (HPLC) buffer: 0.2 *M* phosphate, pH 7.0. Add 0.2 *M* Na_2HPO_4 to 0.2 *M* NaH_2PO_4 to obtain the required pH.

6. *o*-PDM/DMF for Fab′(SH) alkylation: 12 m*M* *o*-PDM in dimethylformamide. Make up just prior to use. Chill in a methylated spirit/ice bath. Caution: *o*-PDM is toxic and should be handled with care.
7. 1 *M*: NTE8 1 *M* NaCl, 0.2 *M* Tris-HCl, pH 8.0, 10 m*M* EDTA.
8. Iodoacetamide: 250 m*M* in 0.2 *M* TE8 and 50 m*M* in 1 *M* NTE8.

2.2. Chromatography Equipment

1. Two chromatography columns packed with Sephadex G25 (*see* **Subheading 2.1**) and equilibrated and run in 50 m*M* AE are required. The first (column 1) should be 1.6 cm in diameter, packed to a height of 25 cm with gel, and pumped at approx 60 mL/h. The second (column 2) should be 2.6 cm in diameter, packed to a height of 20 cm with gel, and pumped at approx 200 mL/h. The columns must be fitted with two end-flow adaptors and water jackets to allow chilling throughout the procedure. Pharmacia K Series columns are ideal. (Cleaning of columns to avoid endotoxin contamination, *see* **Note 9**).
2. Two larger columns packed with polyacrylamide agarose gel (Ultragel AcA44; *see* **Subheading 2.1**) and run in 0.2 *M* TE8 are used for the size exclusion chromatography of the BsAb products. These should be 2.6 cm in diameter, and packed to a height of 80 cm with gel. The two columns should be joined in series using Teflon capillary tubing and pumped at approx 30 mL/h. Chilling is not required at this stage of the preparation, and the columns can be run at room temperature. (Cleaning of columns to avoid endotoxin contamination, *see* **Note 9**).
3. Two peristaltic pumps capable of rates between 15 and 200 mL/h for column chromatography.
4. Chiller/circulator to cool columns. A polystyrene box containing water and crushed ice and a submersible garden pond pump (rate approx 10 L/min) can be used as an alternative to a commercial chiller.
5. UV monitor, chart recorder, and fraction collector.
6. Amicon stirred concentration cell (Series 8000, 50 or 200 mL) with a 10,000 M_r cutoff filter for concentration of products.
7. HPLC system fitted with Zorbex Bio series GF250 column (Du Pont Company, Wilmington, DE) or equivalent gel-permeation column capable of fractionation up to approx 250,000 M_r.

3. Methods

3.1. Preparation of Bispecific F(ab′)$_2$ Derivatives

The method described here is for the preparation of F(ab′)$_2$ BsAb starting with 5–20 mg of each parent F(ab′)$_2$ to obtain 1–8 mg of BsAb product.

1. Use equal amounts of F(ab′)$_2$ from the two parent antibodies. The F(ab′)$_2$ should be in 0.2 *M* TE8 at 5–12 mg/mL in a final volume of 1–3 mL. Keep a 50 µL sample of both F(ab′)$_2$ preparations for HPLC analysis (*see* **Subheading 3.3**).
2. Reduce both parent F(ab′)$_2$ preparations to Fab′(SH) using 1/10 vol F(ab′)$_2$ reducing solution (final concentration 20 m*M* 2-ME). Incubate at 30°C for 30 min and

then keep on ice. Maintain the tempterature at 0–5°C for the rest of the procedure unless stated otherwise.

3. Select the species to be maleimidated (Fab′-A[SH]) (*see* **Note 3**). Remove 2-ME by passing through the smaller Sephadex G25 column (column 1). Collect the protein peak, which elutes after approx 8–10 min, in a graduated glass tube in an ice bath (*see* **Note 4**). Take a 45 µL sample from the top of the peak for HPLC analysis (*see* **Subheading 3.3**). Keep the column running to completely elute 2-ME, which runs as a small secondary peak.
4. When the chart recorder has returned to baseline, load the second Fab′(SH) species [Fab′-B(SH)] onto the column, and separate as for Fab′-A(SH), again taking a sample for HPLC analysis (*see* **Subheading 3.3.**).
5. After the Fab′-B(SH) has been loaded onto the G25 column, the Fab′-A(SH) partner can be maleimidated. Rapidly add a 1/2 vol (normally 4–5 mL) of cold *o*-PDM/DMF to the Fab′-A(SH), seal the tube with Parafilm or similar, and mix by inverting two to three times (*see* **Note 5**). Stand in an ice bath for 30 min.
6. When the Fab′-B(SH) has been collected, connect the larger Sephadex G25 column (column 2) to the chart recorder. After the 30 min incubation, load the Fab′-A(SH)/*o*-PDM/DMF mixture onto this column. Collect the Fab′-A(mal) protein peak (elutes after 8–10 min) (*see* **Note 6**).
7. Pool the Fab′-A(mal) and the Fab′-B(SH). Immediately concentrate in a stirred Amicon concentration cell to around 5 mL, and then transfer to a tube for overnight incubation at 4°C (*see* **Note 7**).
8. During conjugation, in addition to the required BsAb, disulfide bonded homodimers may also form. To eliminate these, after overnight incubation add 1/10 volume 1 *M* NTE8 to the mixture to increase the pH, and then 1/10 vol F(ab′)$_2$ reducing solution to reduce the homodimer disulfide bonds. Incubate at 30°C for 30 min.
9. Alkylate to block sulphydryl groups by the addition of 1/10 vol 250 m*M* iodoacetamide in 0.2 *M* TE8 (*see* **Note 8**). Check the composition of the mixture by HPLC (*see* **Subheading 3.3**).
10. Separate the products on two AcA44 columns run in series. Collect 10–15 min fractions. A typical elution profile is shown in **Fig. 2**.
11. Pool the fractions containing the BsAb product. To minimize contamination, only take the middle two-thirds of the peak. Concentrate and dialyze into appropriate buffer.
12. If required, check the final product by HPLC (*see* **Subheading 3.3**).

3.2. Preparation of Bispecific F(ab′)$_3$ Derivatives

This is as for the preparation of bispecific F(ab′)$_2$ except that the ratio of Fab′(mal) to Fab′(SH) is increased from 1:1 to 2:1 or greater. Therefore, start with at least twice as much of the F(ab′)$_2$ which is to provide two arms of the F(ab′)$_3$ product.

3.3. HPLC Monitoring

For rapid analysis of products during the preparation, an HPLC system is used as described in **Subheading 2.2**. This will resolve IgG, F(ab′)$_2$, and Fab′

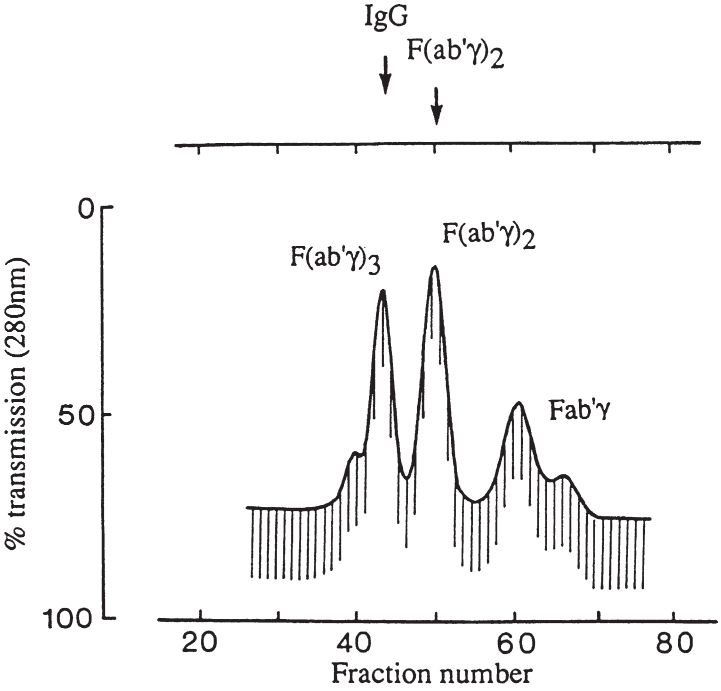

Fig. 2. Chromatography profile showing the separation of parent Fab′ and bispecific F(ab′)$_2$ and F(ab′)$_3$ products on AcA44 columns. In this case, Fab′-A(SH) and Fab′-B(mal) were mixed at a ratio of 2:1 to increase the formation of bispecific F(ab′)$_3$. The unreacted Fab′ fragments and the F(ab′)$_2$ and F(ab′)$_3$ products are indicated. The *arrows* show the points at which protein standards eluted from the same columns.

sized molecules in approx 20 min, and can be performed while the preparation is in progress. The parent F(ab′)$_2$ and the alkylated reaction mixture can be loaded directly onto the column and the eluted product monitored at 280 nm. However, we have found that F(ab′)SH rapidly reoxidizes back to F(ab′)$_2$ while on the column. This can be overcome by alkylating the free SH-groups by the addition of 5 µL 50 m*M* iodoacetamine in 1 *M* NTE8 to the 45-µL sample from the G25 column.

Fab′ will elute from the column later than F(ab′)$_2$ resulting in a shift in the position of the peak on reduction. In most cases >95% of the F(ab′)$_2$ is reduced. Following alkylation and overnight incubation, the reaction mixture typically elutes from HPLC as a triplet, containing a mixture of alkylated FabЭ and Fab′(mal), which elute in a similar postion to Fab′(SH), bispecific F(ab′)$_2$ product, which elutes similarly to the parent F(ab′)$_2$, and a smaller amount of bispecific F(ab′)$_3$, which elutes similarly to IgG.

4. Notes

1. Two SH groups may also be produced by the reduction of the heavy/light chain disulfide bond. However, under the conditions used, this bond is not fully reduced, and any SH groups that are produced are less likely to be available for conjugation ***(1,2)***. This procedure relies on one maleimidated hinge SH-group remaining free for conjugation after the intramolecular cross-linking of adjacent SH-groups with *o*-PDM. It follows that the Fab′ species chosen to be maleimidated must be derived from IgG with an odd number of hinge region disulfide bonds. Of the mouse IgG subclasses, IgG1 and IgG2a (three bonds), and IgG3 (one bond) qualify, whereas IgG2b (four bonds) does not. F(ab′)$_2$ derived from rabbit Ig (one bond) and rat IgG1 (three bonds)can also be employed. However, rat IgG2a and IgG2c both have two, and rat IgG2b has four and so cannot be used as the maleimidated partner.
2. If F(ab′)$_3$ derivatives are required, the number of SH-groups at the hinge of the unmaleimidated partner should be at least two and preferably three, because this determines the number of Fab′(mal) arms that can be conjugated.
3. We have found that a few antibodies give consistently low yields of BsAb when used as the maleimidated partner. If large quantities of a derivative are required, it is worthwhile performing small scale pilot preparations to determine which maleimidated partner gives the optimal yield.
4. It is very important to avoid contamination of the Fab′-A(SH) with 2-ME. In order to minimize the risk, stop collecting when the recorder has returned two-thirds of the way back to the baseline. In order to ensure that Fab′-B(SH) is not contaminated with 2-ME left over from the first run, make sure that it is not loaded until the chart recorder has returned to the baseline.
5. Sometimes the mixture becomes slightly cloudy.
6. To avoid contamination with *o*-PDM/DMF, which elutes as a second large peak, stop collecting when the chart recorder has returned halfway to baseline.
7. To avoid loss of product, slightly over concentrate, then wash the cell with a small volume of chilled buffer.
8. It is very important to add excess iodoacetamide at this stage; otherwise the BsAb derivative can precipitate.
9. To minimise contamination with endotoxin, buffers and reagents are made up using commercially available endotoxin-free water, and glass vessels washed with 0.2 M NaOH and thoroughly rinsed with endotoxin-free water before use. In addition, where possible chromatography columns are cleaned up regularly by running through with 0.2 M NaOH for 30 min

followed by extensive washing with running buffer. *However, please check the manufacturers' instructions to ensure that this is not detrimental to the gel matrix being used*. As both the preparation of the parent F(ab′)$_2$ and the BsAb are multistage processes involving incubation with enzyme and several column chromatography and concentration steps, it is still possible that the product will be contaminated with LPS. To assay for endotoxin, we use Endosafe^R –PTS cartridges (Charles River Laboratories, Charleston, SC) and collect the 'clean product' in an endotoxin-free disposable plastic container. To remove any endotoxin we use EndoTrap^R Red endotoxin removal columns (Lonza, Walkersville, MD); these have a high binding capacity for endotoxin, and can be regenerated and re-used. To avoid re-contamination, minimise manipulations of the "clean" BsAband use endotoxin-free disposable plasticware.

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Antigen Measurement Using ELISA

William Jordan

1. Introduction

The initial description of the enzyme-linked immunosorbent assay (ELISA) almost 30 yr ago ***(1)*** marked a technological advance that has had an immense impact in both clinical diagnostic and basic scientific applications. This assay represents a simple and sensitive technique for specific, quantitative detection of molecules to which an antibody is available. Although there are a huge number of variations based on the original ELISA principle, this chapter focuses on perhaps the two most useful and routinely performed: (1) the indirect sandwich ELISA—providing high sensitivity and specificity and (2) the basic direct ELISA—useful when only one antibody to the sample antigen is available.

1.1. The Direct ELISA

During the indirect sandwich ELISA (**Fig. 1A**), an antibody specific for the substance to be measured is first coated onto a high-capacity protein binding microtiter plate. Any vacant binding sites on the plate are then blocked with the use of an irrelevant protein such as fetal calf serum (FCS) or Bovine serum albumin (BSA). The samples, standards, and controls are then incubated on the plate, binding to the capture antibody. The bound sample can be detected using a secondary antibody recognizing a different epitope on the sample molecule, thus creating the "sandwich." The detection antibody is commonly directly conjugated to biotin, allowing an amplification procedure to be carried out with the use of streptavidin bound to the enzyme horseradish peroxidase (HRP). As streptavidin is a tetrameric protein, binding four biotin molecules, the threshold of detection is greatly enhanced. The addition of a suitable substrate such as 3,3′,5,5′-tetramethylbenzidine (TMB) allows a colormetric reaction to occur in the presence of the HRP that can be read on a specrophotometer with the

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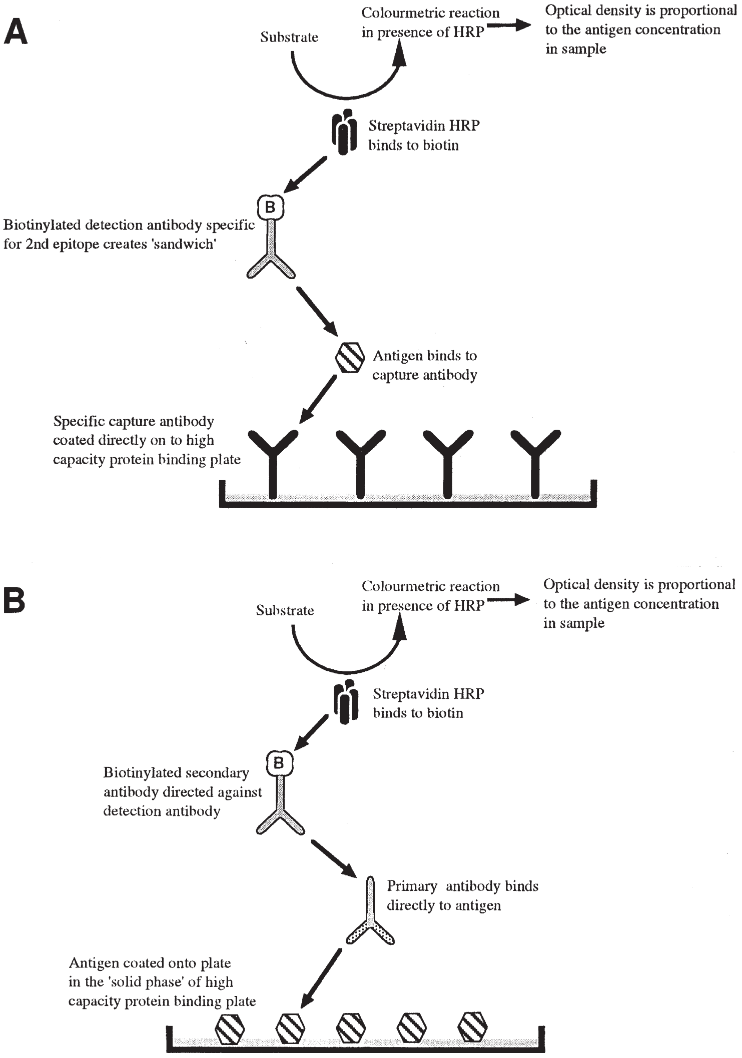
A
Substrate
Colourmetric reaction in presence of HRP
Optical density is proportional to the antigen concentration in sample
Streptavidin HRP binds to biotin
B
Biotinylated detection antibody specific for 2nd epitope creates 'sandwich'
Antigen binds to capture antibody
Specific capture antibody coated directly on to high capacity protein binding plate
B
Substrate
Colourmetric reaction in presence of HRP
Optical density is proportional to the antigen concentration in sample
Streptavidin HRP binds to biotin
B
Biotinylated secondary antibody directed against detection antibody
Primary antibody binds directly to antigen
Antigen coated onto plate in the 'solid phase' of high capacity protein binding plate

Fig. 1. The indirect sandwich ELISA.

resulting optical density (OD) relating directly to the amount of antigen present within the sample.

1.2. The Indirect ELISA

In some cases, however, only one specific antibody may be available, and in such a small quantity that directly conjugating it to biotin would be impractical. In this situation a direct ELISA should be used. During the direct ELISA the sample itself is coated directly onto the microtiter plate and is then detected using the specific antibody. If this antibody is biotinylated, then the procedure can then proceed as in the indirect ELISA; if not, then a secondary biotinylated antibody directed against the species of the detecting antibody itself can be used. **Figure 1B** demonstrates this technique.

To set up a reliable and durable ELISA it is essential to first optimize a number of the parameters mentioned previously. The level of optimization will, of course, depend on exactly what is required from the assay. In some cases a simple "yes-or-no" answer is desired and a simple standard procedure may be sufficient. If, however, high sensitivity is the aim with accurate quantatation of the molecule in question, then carefully setting up the optimal conditions in advance will pay dividends and save a great deal of time in the long term.

2. Materials

1. Antibodies (*see* **Note 1**): For the indirect ELISA, antibody pairs can often be bought commercially and consist of a capture antibody and a biotinylated detection antibody. Both antibodies are specific for the molecule in question, with the detection antibody being directly biotinylated, and able to recognize the sample molecule when it is bound to the capture antibody on the plate. For the direct ELISA, one specific detection antibody is required, preferably biotinylated, but if not a secondary biotinylated antibody specific for the detection antibody is also needed. Aliquot and freeze at –20°C or lower in small, usable quantities with a carrier protein at such as 10% FCS or 1% BSA.
2. Blocking buffer: Phosphate-buffered saline (PBS), pH 7.4, supplemented with either 1% fatty acid free BSA or filtered 10% FCS. If measuring in tissue culture samples, substitute the FCS with protein representing that within the culture conditions, that is, 10% FCS; in effect the sample will have been preabsorbed with this during culture and thus any non-specific binding to the blocking buffer is avoided.
3. Carbonate coating buffer: 8.41 g Na_2HCO_3 in 1 L freshly made PBS. Dissolve and adjust to desired pH with HCl or NaOH. Store for no more than 1 mo at 4°C (*see* **Note 2**).
4. High-capacity protein binding 96-well microtiter plates: There are a large number of suitable makes including Maxisorp (Nunc), Immunoware (Pierce), Immunlon II (Dynatech), and Costar (*see* **Note 3**).
5. PBS–Tween–10% FCS: Add 0.05 mL of Tween 20 to 90 mL of PBS, pH 7.4, and 10 mL of filtered FCS. Make up fresh as required.
6. Plate sealers or cling film wrap.
7. Plate washing apparatus: Adequate washing is a vital element of achieving a successful ELISA. Although a number of automatic plate washers are available, they are expensive and the use of a wash bottle with good pressure is perfectly suitable, although a little more time consuming.
8. Samples/standards: Standards of known amounts are required for positive controls, and for estimation of levels within samples (via comparison to a titration of known

amounts). All standards should be diluted in medium as near as, or identical to that of the sample solution. Standards are best obtained from a reliable commercial source having been mass calibrated and should be frozen in small, concentrated aliquots at −20°C or lower. Repeated freeze–thaw cycles must be avoided.

9. Spectrophotometer: Any suitable microplate reader able to measure absorbance at the appropriate wavelength.
10. Stop solution: 0.5 *M* H_2SO_4.
11. Streptavidin HRP (*see* **Note 4**): Use in accordance to manufacturer's instructions. Sources of streptavidin HRP include Sigma, Biosource, Pierce, and Zymed.
12. Substrate: One-step TMB (Zymed). Although many other substrates are available for HRP, TMB has high sensitivity with a quick development time. OD can be monitored at 650 nm while the color develops, then at 450 nm when the reaction is stopped with H_2SO_4. TMB may also be obtained in a lyophilized state and made up fresh with hydrogen peroxidase.
13. Washing buffer: Add 0.5 mL of Tween 20 to 1 L of PBS, pH 7.4. Make up fresh as required.

3. Methods

3.1. Basic Sandwich ELISA Protocol

1. Dilute the capture antibody to 1 μg/mL is coating buffer, pH 9.5. Add 100 μL to each well of a high-capacity protein binding 96-well microtiter plate.
2. Seal the plate to avoid evaporation and incubate overnight (12–18 h) at 2–8°C.
3. Wash plate: Discard unbound antibody by inverting and flicking the plate over a sink. Fill each well with washing buffer and leave for at least 10 s before discarding once more. Ensure all liquid has been removed between each wash by repeatedly tapping the plate onto clean paper towels. Repeat 3×.
4. Add 200 μL of blocking buffer to each well. Seal plate and incubate for at least 2 h at room temperature.
5. Discard blocking buffer. Wash plate 3× as described in **step 3**.
6. Prepare a titration series of known standards (e.g., in 1.5-mL Eppendorf tubes) diluted in a matrix representing that of the samples (e.g., culture medium or human serum). Include a negative control such as culture medium only. Transfer samples and antigen standards to the ELISA plate in duplicate at 100 μL/well. Seal plate and incubate at room temperature for at least 2 h, or overnight at 4°C for increased sensitivity.
7. Wash plate 3× as described in **step 3**.
8. Dilute biotinylated detection antibody to 1 μg/mL in PBS, pH 7.4. Add 50 μL/well. Incubate at room temperature for 2 h. If problems with nonspecific binding of the biotinylated antibody to the plate occur, dilute the antibody in PBS–Tween–10% FCS rather than just PBS.
9. Wash plate 3× as described in **step 3**.
10. Dilute streptavidin HRP according to manufacturer's instructions. Add 100 μL/well. Incubate at room temperature for 30 min. Again, if problems with nonspecific binding of the biotinylated antibody to the plate occur, dilute the antibody in PBS-Tween-10% FCS rather than just PBS.

11. Wash plate 4× as described in **step 3**.
12. Add 100 µL/well of “one step” TMB. Allow color to develop for 10–60 min (20 min is usually sufficient). OD may be monitored at this stage at 650 nm as the color develops.
13. Add 100 µL of 0.5 *M* H_2SO_4 to each well to stop the reaction. Read OD at 450 nm.
14. Estimate amount of antigen within samples by comparing ODs to those of known standards.

3.2. Direct ELISA Protocol

1. Make serial dilutions of samples from neat to 1 : 16 in coating buffer, pH 9.5. Add 100 µL to each well of a high-capacity protein binding microtiter plate. Also set up wells with antigen standards and a negative control diluted in the same coating buffer.
2. Seal the plate with an acetate plate sealer or cling film wrap to avoid evaporation and incubate overnight (12–18 h) at 2–8°C.
3. Wash plate: Discard unbound antibody by inverting and flicking the plate over a sink. Fill each well with washing buffer leave for at least 10 s and discard once more. Ensure all liquid has been removed between each wash by repeatedly tapping the plate onto clean paper towels. Repeat 3×.
4. Add 200 µL of blocking buffer to each well. Seal plate and incubate for at least 2 h at room temperature.
5. Discard blocking buffer. Wash plate 3× as described in **step 3**.
6. Dilute detection antibody to 2 µg/mL in PBS. Add 50 µL/well. Incubate at room temperature for 2 h. If the antibody is biotinylated proceed to **step 9**.
7. Wash plate 3× as described in **step 3**.
8. Add secondary antibody (100 µL, 2 µg/mL) specific for the detection antibody (i.e., if the detection antibody is mouse, use biotinylated rabbit anti-mouse Ig).
9. Wash plate 3× as described in **step 3**.
10. Dilute streptavidin HRP according to the manufacturer’s instructions. Add 100 µL/well. Incubate at room temperature for 30 min.
11. Wash plate 4× as described in **step 3**.
12. Add 100 µL/well of “one-step” TMB. Allow color to develop for 10–60 min (20 min is usually sufficient). OD may be monitored at this stage at 650 nm, as the color develops.
13. Add 100 µL of 0.5 *M* H_2SO_4 to each well to stop the reaction. Read OD at 450 nm.
14. Estimate amount of antigen within samples by comparing ODs to those of known standards of sample.

3.3. Optimization of ELISA

At this stage the ELISA may be more than adequate, although optimization of the following parameters is usually required:

1. pH of carbonate coating buffer (pH 7.0–pH 10.0). Also try PBS at pH 7.4.
2. Concentration of capture antibody (0.5–10.0 µg/mL).

3. Concentration of secondary antibody (indirect ELISA, 0.5–4.0 μg/mL).
4. Concentration of biotinylated detection antibody (0.1–2.0 μg/mL).
5. Concentration of streptavidin–HRP conjugate (usually between 1 : 1000 and 1 : 10,000).

4. Notes

1. The quality of the antibodies used is perhaps the most important aspect in seting up a good ELISA. Antibodies need to have a high affinity for the sample to be measured, reducing the chance of being "washed off" during the assay. The indirect ELISA relies on two specific antibodies and thus the increased specificity increases sensitivity of the assay by reducing background.
2. The pH of the coating buffer can have a great effect on the amount of antibody that will bind to the plate and thus to the ELISA as a whole. Basically, a higher pH will result in more antibody binding but may have a detrimental effect on its immunoreactivity. Thus a pH must be found that is suitable for the antibody in question, and this can vary dramatically. When beginning optimization of the assay, test a range of carbonate buffers from pH 7.0 to 10.0 as well as PBS, pH 7.4. We usually find a carbonate buffer pH of 9.5 gives good results. In some cases we have found commercially available coating antibodies recommended by the manufacturer to be adsorbed onto the plate at pH 7.4 to be far more effective at higher pH values, improving the sensitivity by up to 10-fold. This can allow the coating concentration to be vastly reduced, creating an extremely cost-effective assay.
3. There can be a significant difference between the protein binding capability of different makes, and even batches of microtiter plates. Some appear to be extremely good for binding antibodies, whereas others more useful for other proteins. The only real way to choose a suitable plate is by trial and error, or using a recommended make known to bind the protein you intend to coat.
4. Although the HRP–TMB system represents a good, reliable, and sensitive combination, HRP has a number of alternative substrates that can be used *(2)*. These include o-phenylene diamine (OPD) or 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid (ABTS). There are also a number of options for the enzyme used other than HRP, such as alkaline phosphatase (AP) which can be used in combination with the substrate *p*-nitrophenyl phosphate (p-NPP) or phenolphthalein monophosphate (PMP). The choice of enzyme–substrate system depends on a number of factors including price, sensitivity, and whether a filter is available for the substrate specific wavelength to be measured.

5. The use of biotinylated secondary antibodies (or detection antibody in the indirect sandwich ELISA) in conjunction with enzyme-conjugated streptavidin (or avidin, extravidin, etc.) both increase sensitivity and save time in that a further step is eliminated from the assay and therefore another step of optimization is eliminated.
6. If the major aim of the ELISA is to obtain quantitation of substances present in extremely low concentrations a number of adaptations to the technique can be used. Such techniques often use AP enzyme systems which can be utilized, for example, to lock into a circular redox cycle producing an end product such as red formazan which is greatly amplified in comparison to standard amplification methods ***(3)***. Chemiluminescent amplified ELISA principles have also been shown to give very high sensitivity ***(4)*** and can be optimized to measure as little as 1 zeptomol (about 350 molecules!) of AP ***(5)***. Although extremely sensitive, such techniques are time consuming to set up and optimize, and are far more expensive than the simple colormetric ELISAs described in this chapter.
7. In some cases, molecules present in a sample are masked by the solution that they are in. This problem can sometimes be solved by diluting the samples in PBS–Tween–10% FCS. If this is performed, remember to make similar adjustments to the solution used for the standards. Possible interference molecules within samples such as soluble receptors for the antigen can also cause a problem. Commercially available matched antibody pairs for molecules should have been pretested and guaranteed against being affected by such problems.

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Enhanced Chemiluminescence Immunoassay

Richard A. W. Stott

1. Introduction

Chemiluminescence results from reactions with a very high energy yield, which produce a potentially fluorescent product molecule; reaction energy passed to the product may result in an excited state and subsequent production of a single photon of light. The light yield is usually low, but can approach one photon per molecule in bioluminescent reactions catalyzed by dedicated enzymes.

A wide variety of immunoassay systems that use chemiluminescence or bioluminescence have been developed with the aim of detecting low concentrations of biologically active molecules. Even when emission efficiencies are <1%, chemiluminescence is a sensitive label detection method compared with isotopic methods in which very large numbers of molecules must be present for each detected disintegration (e.g., about 1×10^7 atoms of ^{125}I give 1 count/s). The production of light against a low background permits detection of small numbers of reacting molecules by measuring total light output. Luminescent emissions can be measured over a range of at least six orders of magnitude by all but the simplest luminometers. This is in marked contrast to fluorescent or spectrophotometric detection of reaction products, where sensitivity and instrument linear range are limited by the stability of light sources and wavelength selection. For example, a good spectrophotometer may achieve a linear range slightly greater than three orders of magnitude.

Directly labeled chemiluminescent systems produce <1 photon/label and require complex chemical synthesis to produce each new labeled molecule ***(1, 2)***. In contrast, chemiluminescent detection of enzyme labels combines the advantages of a high specific activity label with the convenience of relatively simple coupling chemistries, which use commercial reagents. A number of enzyme labels can be detected via chemiluminescent or bioluminescent

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reactions, including β-galactosidase *(3)*, alkaline phosphatase *(4)*, peroxidase, luciferin and a variety of enzymes indirectly linked via production of ATP or NADH *(5)*. Systems that use alkaline phosphatase and peroxidase have the additional advantage of commercial availability of a wide range of labeled molecules and complete assay kits suitable for adaptation to luminescent detection.

Enhanced chemiluminescence is based on the reaction of luminol (3-aminophthalhydrazide) with an oxidizing agent, such as hydrogen peroxide or sodium perborate. This reaction is catalyzed by metal ions at high pH, resulting in emission of blue light (emission peak about 425 nm). At lower pH, the reaction is catalyzed by hemecontaining enzymes, such as horseradish peroxidase, catalase, cytochrome C, and hemoglobin (**Fig. 1**). However, the light output is low with a half-life of a few seconds. The presence of any one of a series of "enhancer molecules" increases the light emission from horseradish peroxidase by 1000-fold or more, and alters the kinetics so that a steady glow is produced lasting several hours *(6, 7)*. Microparticles, plastic beads, plastic tubes, microtiter plates, membranes, and plastic pins have all been used successfully as solid supports in a wide range of enhanced chemiluminescence assays, including competitive immunoassays, immunometric assays, and RNA and DNA binding assays *(6, 8, 9)*.

In common with all other sensitive detection systems, maintenance of the label enzyme in its active state is important. The precautions detailed in **Notes 1–3** should be observed to maximize the sensitivity achieved. Reagents for enhanced chemiluminescence can be prepared in the laboratory or are available commercially (*see* **Note 4**). The purity of the substrate solution is important in achieving maximum sensitivity. Therefore, the precautions detailed in **Notes 5–7** should be followed if preparing substrate solutions. The free base form of luminol undergoes rearrangement to a mixture of luminol and a series of contaminants. Therefore, luminol should be purified by recrystallization as the sodium salt before use (*see* **Note 8**).

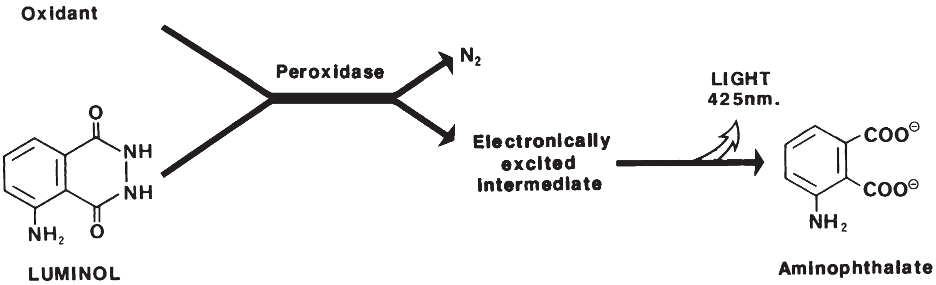

Fig 1. Chemiluminescent oxidation of luminol by peroxidase.

1.1. Light Measurement Instruments (Luminometers)

Commercial luminometers range from low-cost manual single tube instruments to fully automated high-capacity machines and have been reviewed previously ***(10)***. However, application-specific requirements are rarely discussed, and the first-time user will require some guidance in matching an instrument to the chemistry or chemistries to be used.

There is normally no requirement for wavelength selection, because very few reactions produce significant light output. Therefore, a simple luminometer can consist of a detector and some means of presenting a sample or samples in a light-tight compartment. There may also be a system for adding reagents to the sample while in the chamber. The detector is usually a photomultiplier tube for sensitive instruments, but can be a photodiode in a portable instrument ***(7, 11)*** or photographic film, if a semiquantitative result is sufficient ***(2, 12)***.

Luminometers designed for use with short-lived reactions have complex high-precision reagent injection systems. There may also be a short measurement prior to reagent injection to correct for background owing to light leaks, phosphorescence, and scintillation from sample tubes. Photon-counting and cooled detectors may also be used to achieve maximal light-detection sensitivity. None of these features is essential for use with enhanced chemiluminescence.

Relatively high light intensities and prolonged emission are produced by enhanced chemiluminescence detection of peroxidase and the substituted dioxetane-based detection reactions for alkaline phosphatase or β-galactosidase ***(3, 4)***. These reactions require a short stabilizing time before reading and are conveniently performed by adding the reagents before the sample reaches the measuring position. This can only be done if any preinjection blank measurement can be disabled. It is also practical to handle large numbers of samples using a timed reagent addition outside the instrument, completely eliminating the need for automatic reagent handling. High light output can lead to "pulse pileup" in photon-counting electronics, resulting in nonlinearity and eventually zero apparent signal ***(13)***. Linearity can be improved via mathematical correction for the dead time or insertion of a neutral density filter.

Although light output from individual transparent microtiter wells can be measured in a luminometer designed for tubes, it is more convenient to use opaque microtiter plate wells and one of the purpose-designed readers. Prolonged light output makes it possible to start the reaction outside the instrument in the same pattern as the light emission is read. However, the presence of other glowing wells introduces the possibility of light carryover into the well being read. Carryover is important if the dynamic range of light emissions from a plate is expected to be higher than three orders of magnitude; in this case, there is a risk of false-positive results owing to a fraction of a percent of the emission from a very high sample being transmitted to a low one as much as three positions away (**Table 1**).

Table 1
Carryover from a Single Glowing Well in a Rigid[a] Black Microtiter Plate Measured Using a Prototype Luminometer

0.02%	0.01%	0.007%	ND	ND
0.04%	0.035%	0.018%	0.005%	ND
0.71%	0.075%	0.034%	0.007%	ND
Source	0.1%	0.03%	0.007%	ND
0.045%	0.034%	0.025%	0.006%	ND
0.03%	0.025%	0.013%	0.005%	ND
0.009%	0.009%	0.006%	ND	ND

[a] Data represent the mean of several readings obtained for empty wells expressed as a percentage of the mean light output of the source well. Positions marked ND gave readings that were not significantly different from the photomultiplier background.

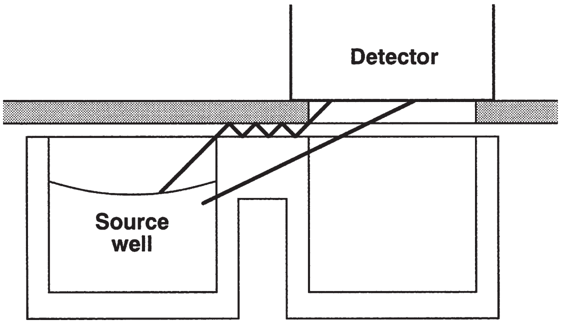

Fig. 2. Origin of light carryover between wells of an "opaque" microtiter plate.

Both white and black microtiter plates are available in single-well, strip, and plate formats. The plastic is made opaque by incorporating colored particles into transparent plastic. Therefore, some light can pass through the plastic. The light transmission differs considerably between formats and individual manufacturers. However the greatest carryover occurs via external reflection from the shiny top surface (**Fig. 2**). Carryover is least for black plastic, although the loss of reflection within the well also reduces the signal available to the detector. Multiwell strips have higher carryover along the strip than between adjacent strips owing to the plastic web that links the wells. Similarly, individual wells have lower carryover than joined ones.

Carryover varies considerably between instruments, but it is particularly low if the instrument has an antireflection mask between the plate and detector. Individual instruments should be assessed for carryover using the type of plates that will be used with it. The pattern of carryover should be determined by reading light output from all wells of a plate containing a single glowing well with a light output, which represents the highest expected from the assay. The location of this well may affect the results, and carryover should be assessed using each corner well and one close to the center of the plate.

2. Materials

1. High-quality deionized water (*see* **Note 3**).
2. Luminol stock solution: 1.25 m*M* luminol in 0.1 *M* Tris-HCl, pH 8.6. Store at 4°C in the dark. Make up fresh each week. Luminol should be recrystallized as the sodium salt before use (*see* **Note 8**).
3. Hydrogen peroxide: 30% (w/v). Store at 4°C.
4. p-Iodophenol stock solution: p-iodophenol, 1 mg/mL in dimethyl sulfoxide (DMSO). Make up fresh each day.
5. Microfluor "B" microtiter plates (Dynatech Laboratories, Chantilly, VA).
6. Coating buffer: 0.1 *M* glycine, pH 8.8. Adjust pH using NaOH. Store at 4°C, and make up fresh each week.
7. Rabbit anti-α fetoprotein (AFP) (Dako [Glostrup, Denmark]).
8. Phosphate-buffered saline (PBS), pH 7.2: 0.14 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, and 8.1 m*M* Na_2HPO_4. Store at 4°C, and make fresh each week.
9. Blocking solution: PBS containing Bovine serum albumin (BSA) 0.1% (w/v). Make up fresh each day.
10. PBS-Tween: PBS containing Tween-20, 0.05% (v/v). Make an additional batch using high-quality deionized water (*see* **Note 3**). Store at 4°C, and make fresh each week.
11. Assay diluent: PBS-Tween containing BSA 0.5% (w/v). Make up fresh each day.
12. Working standards concentration range 0–800 ng/mL made fresh for each assay batch by serial dilution of stock standard using normal human serum. Stock standard (1600 ng/mL) is made by diluting AFP standard serum (Dako cat. no. X900 or Boehring Diagnostics [Westwood, MA] cat. no. OTOD 02/03) in human serum containing 0.05% (w/v) sodium azide. Store frozen as 1-mL aliquots.
13. Peroxidase-conjugated anti-AFP (Dako cat. no. P128) diluted 1/1000 in assay diluent. Make up fresh for each assay batch.
14. Working enhanced chemiluminescence substrate solution (*see* **Note 9**)—either:
    a. p-iodophenol-enhanced substrate: 1.25 m*M* luminol, 4 μ*M* p-iodophenol, 2.7 m*M* H_2O_2. Add 15 μL of stock hydrogen peroxide and 40 mL of p-iodophenol stock solution to 50 mL of stock substrate: solution, and mix well. Make up daily using high-quality deionized water (*see* **Note 3**), and store in the dark when not in use, or
    b. p-Hydroxycinnamic acid-enhanced substrate: 1.25 m*M* luminol, 30 μ*M* p-hydroxycinnamic acid, 2.7 m*M* H_2O_2. Add 15 mL of stock hydrogen peroxide and 1 mg of p-hydroxycinnamic acid (alternative names: p-coumaric acid or 4-hydroxycinnamic acid, e.g., Aldrich [Gillingham, UK]) to 50 mL of stock substrate solution. Mix for 30 min before using. Make up daily using high-quality deionized water (*see* **Note 3**), and store in the dark when not in use.

3. Method

1. Coat the wells of a microtiter plate with 100 mL of anti-AFP (1/1000 in coating buffer). Allow the protein to bind for either 2 h at room temperature or overnight at 4°C (*see* **Note 10**).
2. Empty the wells, and wash off any unbound antibody by filling each well with PBS and shaking the plate to re-empty. Repeat the wash and block unbound sites by incubating with 200 mL/well of blocking solution for 30 min at room temperature.
3. Empty the wells, and wash the plate twice (as described in **step 2**) with PBS-Tween to remove any unbound albumin.
4. Prepare 1/20 dilutions of samples, standards, and quality control specimens using assay diluent, and add 150 µL to each of the microtiter plate wells. Cover the plate with plastic film, and incubate at 37°C for 1 h.
5. Empty the wells, and wash the plate three times with PBS-Tween. Shake the plate over a sink to ensure complete removal of wash solution. Add 150 µL of working conjugate to each well, cover, and incubate at 37°C for 1 h.
6. Empty the wells, and wash the plate three times with PBS-Tween made using high-quality deionized water (*see* **Note 3**).
7. Add 150 µL of enhanced chemiluminescent substrate to each well in the same order and preferably with the same timing as used by the plate reader. Allow at least 2 min for the light output to stabilize before reading the plate.
8. Obtain unknown specimen results either by reading off a plotted calibration curve or use a computer program to calculate from a fitted curve.

4. Notes

1. Peroxidase is inactivated by anions and certain antimicrobial agents, including azide, cyanide, and thiomersal. Antimicrobial agents may be present in concentrated enzyme label solutions and assay buffers at typically active concentrations, but must not be present in wash solutions or substrate. The latter reagents must be freshly made each day from concentrated stocks.
2. Powerful oxidizing or reducing agents may interfere with any peroxidase detection reaction by inactivating peroxidase, oxidizing the substrates or reducing the oxidants in the reagents. There are potentially many of these in the laboratory environment, including chlorine in water, disinfectants, paper dust, laboratory coats, skin, and so forth. Care should be taken to avoid contamination of individual assay wells or equipment.
3. For best possible sensitivity, the final assay wash (**step 6**) and all substrate reagents should be made up in water of the highest possible purity. Trace contamination with bacteria, algae, organic compounds, and chlorine is a particular problem. Laboratory-grade distilled or reverse osmosis water should be further treated using a deionization cartridge. The water plant must be well maintained to avoid bacterial growth in deionization columns,

plumbing, and storage tanks. Alternatively, commercial HPLC-grade water has been found to be satisfactory.

4. Amerlite signal reagent (Johnson and Johnson Clinical Diagnostics) is supplied as separate bottles of buffer and substrate tablets. One "A" and one "B" substrate tablet (cat. no. LAN.4401) are dissolved in each bottle of substrate buffer (cat. no. LAN. 4402) prior to use. This reagent is stable for a day at room temperature providing it is kept in the dark glass bottle.
5. In order to ensure stable concentrations, the anhydrous form of sodium luminol is preferred molecular weight 199.1. This should be stored over silica gel in the dark. Luminol solutions should be stored in the dark at 4°C. Stock solutions must be made up at least weekly and working substrate daily.
6. DMSO is a colorless, odorless compound. However, it is hygroscopic and acquires an onion-like smell. DMSO in this state has been found to be inhibitory in the enhanced chemiluminescent reaction. Therefore, the highest available grade should be purchased in small amounts and carefully stored to minimize water uptake.
7. Hydrogen peroxide is a powerful oxidant, but gradually loses activity. The highest available grade should be purchased in small amounts, and stored at 4°C.
8. Luminol is available as the free base under alternative chemical names from several chemical suppliers, including:

   a. 5-Amino-2, 3-dihydro-1, 4-phthalazinedione (Sigma [St. Louis, MO]).
   b. 3-Aminophthalhydrazide (Aldrich).

   There is considerable batch to batch variability in commercial luminol. The purity of the original material is only important in determining how many recrystallization steps are required. Recrystallization gives a consistently high activity product and may be performed as follows (for further details, *see* **ref.** *7*):

   a. Dissolve luminol in 5% (w/v) sodium hydroxide at room temperature until close to saturation (about 200 g/L). The color of this solution will depend on the original luminol. Cool the solution in an ice bath and allow to crystallize for 4 h. Recover the sodium luminol crystals using suction filtration on a glass fiber filter disk (Whatman GFA or similar), and wash using a small volume of ice-cold 5% sodium hydroxide. The crystals should be white or only slightly discolored. Sodium luminol should be recrystallized at least twice after a white product is obtained.
   b. Dissolve the sodium luminol in a minimum volume of 5% sodium hydroxide at room temperature. Allow to crystallize on an ice bath for 18 h. A refrigerator can be used as an alternative, although crystallization may be slower and yield may be low. Recover the crystals by filtration as above.

The initial crystalline form is the hexa-hydrate, which converts to an anhydrous powder on drying over silica gel. Sodium luminol is stable to heat (melting point >400°C), but Sodium luminol has recently become available from Sigma (cat. no. A 4685). The author has no experience with this product.

9. The substrate solutions detailed here (**Subheading 2, item 14**) are essentially interchangeable with no sensitivity advantage for either. Both systems are optimized for a reasonably steady light output at a peroxidase concentrations typically encountered in immunoassays. Use of final peroxidase activities that are markedly higher will result in declining light output owing to substrate exhaustion. This cannot be avoided by alteration of the reaction conditions. Slight reduction in enhancer concentration may give more stable light output for assays with atypically low peroxidase activity.
10. For best results, plates should be coated with the IgG fraction of an antiserum, this can be conveniently prepared using caprylic acid precipitation (*see* Chapter 180 and **ref. *15***). Where this is not possible, indirect capture may be used, such as antispecies antibody on the plate, or the streptavidin–biotin system. Any indirect capture system must be compatible with the final label, e.g., labeled antigen or different species antisera with no cross-reaction with the indirect coating antibody.
11. Safety data (from **ref. *16***):
    a. Luminol (commercial-grade): Irritating to eyes, respiratory system, and skin. No specific information is available for pure luminol or for the sodium salt.
    b. Hydrogen peroxide: Contact with combustible materials may cause fire. Causes burns. Keep in a cool place. After contact with skin, wash immediately with plenty of water.
    c. DMSO: Irritant to eyes, skin, and respiratory system. Harmful by inhalation, skin contact, and if swallowed. May cause sensitization by inhalation or skin contact.
    d. *p*-Iodophenol: Irritant to eyes, skin, and respiratory system.
    e. *p*-Hydroxycinnamic acid: Irritant to eyes, skin, and respiratory system.

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Immunoprecipitation and Blotting

The Visualization of Small Amounts of Antigens Using Antibodies and Lectins

Stephen Thompson

1. Introduction

Immunoprecipitation and blotting both use antibodies (normally, but not exclusively monoclonal antibodies) to detect and quantitate specific protein antigens in complex cellular or protein mixtures. Immunoprecipitation has an advantage in that the antigens are allowed to react with the antibodies in their native conformation prior to their subsequent separation and quantification. A further advantage is that a protein at a very low concentration can be concentrated from the relatively large volume of 1–2 mL. The major disadvantage is that the proteins normally have to be radiolabeled to facilitate their detection. In Western blotting the proteins do not have to be labeled, but they have to be separated by electrophoresis in polyacrylamide gels prior to their transfer to either nitrocellulose, polyvinyldifluoride (PVDF), or nylon membranes. This seriously restricts the size of the sample, and hence the protein antigen has to be present at higher concentrations. A further disadvantage is that the antigen is not normally in its native conformation when it reacts with the antibody, because the electrophoresis usually being carried out in the presence of sodium dodecyl sulfate (SDS) to maximize the resolution of the separated proteins. If an antibody has a lower affinity for an antigen, it may well immunoprecipitate an antigen but not react with it on a Western blot. This is the reason why some workers slot-blot their protein mixtures rather than separate them by electrophoresis. This maintains their native conformation. The main problem encountered here is that crossreactions of the primary antibody with all the other proteins in the mixture can outweigh the antigen-specific binding. An unlabeled antigen is often therefore prepurified using an immobilized lectin or even immunoprecipitation itself

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prior to its quantitation on a slot-blot. Such a combination of lectin and antibody techniques has tremendous potential both in more precise analysis of cellular antigens and in the characterization of disease progression.

This chapter therefore discusses the following major steps involved in immunoprecipitation:

1. The labeling and lysis of cells.
2. The formation of antibody–antigen complexes.
3. The removal and separation of the complexes.
4. The quantitation of the separated antigens.

This is followed by a shorter second section discussing Western and slot-blotting. The actual processes involved in Western blotting are not covered in great detail. This has recently been excellently reviewed ***(1)***. However, common practical problems are addressed.

Finally, a third and final section then discusses the use of lectins in both of the above techniques. This section is included because post-translational glycosylation of proteins is becoming increasingly recognized as an important factor in determining the course of many diseases including cancer.

2. Materials

All chemicals were of the purest grade possible (analar grade). All enzymes and second-layer antibodies were purchased from Sigma unless otherwise stated.

2.1. Immunoprecipitation

1. Radioactive amino acids (20–200 µCi), sugars or $^{32}PO_4$.
2. Minimal essential medium (MEM) depleted of the appropriate amino acid.
3. Fetal calf serum (FCS).
4. Phosphate-buffered saline (PBS).
5. Glucose: 0.5 *M* in PBS.
6. Lactoperoxidase: 1 mg/mL in PBS (stored in frozen aliquots).
7. KI: 100 µ*M* in PBS.
8. Glucose oxidase: 10 µL in 10 mL PBS (make up just before use).
9. $Na^{125}I$: 100–200 µCi.
10. Cell lysis buffer: 25 m*M* Tris-HCl buffer, pH 8.0, 150 m*M* NaCl, 1 m*M* $MgCl_2$ (TBS), 0.5–1.0% Nonidet P-40 (NP-40), 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF; made up as 100 m*M* stock solution in ethanol).
11. CNBr-activated Sepharose beads (Pharmacia).
12. Primary antibody.
13. LP3: 3 mL round-bottomed plastic tubes.
14. Primary monoclonal or polyclonal antisera
15. Protein A-Sepharose beads: washed in TBS to remove preservatives and kept as a 50% suspension.

16. Second-layer sheep or rabbit polyclonal anti-antibody if your primary antibody is a mouse or rat antibody (*see* **Note 9**).
17. SDS: 5–10%.
18. Light-proof film cassettes.
19. X-ray film.
20. Flash gun with filters to presensitize the film.
21. Calcium tungstate scintillation screens (enhanced autoradiography).
22. 2,5-Diphenyloxazole (PPO).
23. Dimethylsulphoxide (DMSO, fluorography).
24. Gel drier.
25. Scanners or densitometers.

2.2. Blotting

1. Nitrocellulose or nylon membranes.
2. Polyoxyethylenesorbitan monolaurate (Tween-20).
3. Apparatus to suck the samples onto a membrane under vacuum (slot-blotting).

2.3. Precipitation and Blotting with Lectins

1. Lectins coupled to Sepharose beads: commercially available or made in an identical procedure to that given for antibodies in **Subheading 3.1.2.1.**
2. Labeled cellular antigens or complex protein mixtures.
3. Dioxigenin (DIG)-coupled lectins (Boehringer Mannheim, Germany).
4. Alkaline phosphate (AP)-labeled anti-DIG antibody second layer.
5. Proteins with known glycosylation structures as positive and negative controls.
6. Biotinylated lectins.
7. Anti-biotin-AP or avidin-AP second layer.

3. Methods

3.1. Immunoprecipitation

3.1.1. The Labeling and Lysis of Cells

There are two main methods of labeling cellular antigens: (1) metabolic labeling and (2) cell surface labeling. Metabolic labeling is normally carried out for at least 16 h in an attempt to label all the cellular proteins, even those with low turnover rates. Cell surface labeling allows an accurate analysis of the surface of a cell at any given time. Metabolic labeling is performed as follows:

1. Add approx 20 µCi/mL ^{35}S, ^{14}C, or ^{3}H amino acids to near confluent cell cultures for 16–18 h in MEM depleted of the appropriate amino acid ***(2,3)***. A typical flask would contain between 5×10^5 and 10^7 adherent cells (*see* **Notes 1–3**).
2. Wash adherent cells once with PBS.
3. Remove cells by treatment with 0.02% EDTA or EGTA for 5–10 min followed by two further washes with PBS (*see* **Note 4**).
4. Wash nonadherent cells three times with PBS to remove surplus radioactivity.

Many techniques have been developed to label the surface of cells, but those utilizing radioactive iodine have proved to be the most popular, probably because of the ease of detection of radioactive iodine in labeled proteins after their separation in polyacrylamide gels. The most commonly used technique is the lactoperoxidase-catalyzed iodination procedure using H_2O_2 generated by the glucose–glucose oxidase system ***(4)***. I have found this technique to be very reliable ***(5–7)***, and it can also be used to iodinate protein mixtures with a very high efficiency (around 95%). The procedure is given below.

1. Wash adherent cells once with PBS.
2. Remove cells by treatment with 0.02% EDTA or EGTA for 5–10 min followed by two further washes with PBS.
3. Resuspend the cells immediately in 1 mL PBS.
4. Add 10 µL glucose, 10 µL glucose oxidase, 5 µL KI, 10 µL lactoperoxidase, and 1–2 µL $Na^{125}I$ (100–200 µCi) (*see* **Subheading 2.1.** and **Note 5**).
5. Leave to react for 20–30 min at 37°C.
6. Wash away any unbound ^{125}I by three further washes with PBS containing 5 µ*M* KI (*see* **Subheading 2.1** and **Note 5**).

Many procedures can be used to lyse labeled cells. These all utilize isotonic buffers with pH values from 7.4 to 8.0 containing 0.5–1.0% nonionic detergent to solubilize the cell membranes. Some workers include EDTA at concentrations up to 10 m*M*, whereas others prefer to add Ca and Mg ions. Cell lysis buffer pH8.0, as described in **Subheading 2.1**, works well and will give good immunoprecipitates with most antibodies from cell lysates solubilized in this buffer. However, antibodies will often not give immunoprecipitates if PBS or Tris buffers at pH 7.4 with the same additives are used as lysis buffers.

1. Collect labeled cells by centrifugation.
2. Solubilize the cell pellet in 500 µL to 1 mL of the cell lysis buffer for 30 min at 4°C with repeated vortexing (*see* **Note 6**).
3. Separate the solubilized components from residual cellular debris by microcentrifugation at 13,000 *g* for 10 min. The solubilized extracts can be used immediately or can be stored frozen at −70°C until required.

3.1.2. Formation of Antibody–Antigen Complexes

Antigens can be immunoprecipitated by direct or indirect methods. The direct method uses the antibody directly coupled to Sepharose beads. In the indirect method the protein mixture is incubated with the antibody, and then the antigen–antibody complexes are removed using Protein A immobilized on Sepharose beads. The indirect method is more commonly used, as the antibody binding is not constrained by its immobilization to beads.

3.1.2.1. Direct Precipitation

The antibody has to be bound to activated-Sepharose beads before it can be used. This is a very simple procedure:

1. Place 0.3 g of CNBr-activated Sepharose in 100 mL of 1 m*M* HCl and allow to swell (to give approx 1 mL of beads).
2. Decant the clear supernatant after 30 min.
3. Gently resuspend the beads in another 100 mL of 1 m*M* HCl to remove the preservatives.
4. After 1 h decant the supernatant again (by suction).
5. Wash the beads rapidly in 10 mL 0.1 *M* sodium bicarbonate and pack by minimal centrifugation (30 s at 500 *g*).
6. Add immediately 1–2 mg (1 mg/mL in bicarbonate) of antibody to 1 mL of beads and mix gently overnight at 4°C.
7. Leave the beads to settle out. Remove the supernatant and measure its absorbance. The OD of the supernatant should be close to zero if the coupling has worked.
8. Add 5–10 mL of a 0.2 *M* glycine solution for 2 h to block residual active groups.
9. Wash the antibody-coated beads twice in 0.1 *M* bicarbonate and PBS. They are then ready for use.
10. For immunoprecipitation, add 25–50 µL of beads (50–100 µL of a 50% bead suspension) to a LP3 tube and add up to 0.5 mL of cell lysate or protein mixture.
11. Leave with gentle mixing for 1 h at room temperature for complexes to form.
12. Wash away unbound proteins (5 × 2 mL; *see* **Note 7**).
13. Dissociate the immune complexes from the beads (see below).

3.1.2.2. Indirect Precipitation

1. For indirect immunoprecipitation add the antibody to up to 1 mL of cell lysate (more often 100–200 µL) and incubate the mixture for 30–40 min at room temperature for anti-gen–antibody complexes to form (*see* **Note 8**).
2. Add 50 µL of a 50% suspension of Protein-A Sepharose to each sample to bind to the antigen-antibody complexes (*see* **Note 9**).
3. After a further 1 h of incubation with frequent gentle mixing, wash the beads five or six times, by gravity or very gentle centrifugation (*see* **Note 7**), with 2.5 mL TBS/NP-40 to remove unbound proteins.

3.1.3. Removal and Separation of the Complexes

1. Remove the immunoprecipitates from the small Sepharose-bead pellets by the direct addition of 50 µL of double-strength SDS-PAGE sample buffer. The ionic detergent totally disrupts the antigen–antibody complexes (*see* **Note 10**).
2. Harvest the solubilized antigen in the supernatant after microcentrifugation (*see* **Note 11**).
3. Add 5% (v/v) 2-mercaptoethanol to reduce the sample.
4. Boil for 5 min to ensure complete solubilization of the proteins.

5. Separate the immunoprecipitates by SDS-PAGE. The discontinuous system of Laemmli ***(5,8)*** gives the best resolution of proteins.

3.1.4. Quantitation of the Separated Antigens

Separated antigens are normally quantitated by autoradiography or fluorography of the dried polyacrylamide gels followed by densitometry of the developed X-ray film.

3.1.4.1. AUTORADIOGRAPHY

This is the simplest procedure, as the polyacrylamide gel is simply dried onto filter paper and placed directly in contact with X-ray film. It is not very sensitive, with bands needing to contain more than 1500 dpm of ^{35}S or ^{14}C to allow their detection in 24 h. Much lower levels of ^{125}I or ^{32}P can be detected owing to their stronger γ and β emissions. Their irradiation is so strong it penetrates completely though the X-ray film. This has allowed the development of an ultrasensitive sandwich detection technique in which a sensitized X-ray film is placed between the dried gel and a calcium tungstate intensifying screen ***(10)***. Emissions pass through the X-ray film and hit the screen, and multiple photons of visible light are emitted that superimpose a photographic image on top of the autoradiographic image. The X-ray film is preexposed to a brief flash of light (*see* **Note 12**), and autoradiography is carried out at −70°C to maximize the detection of the emitted photons ***(10)***.

An indirect autoradiograph of pp60^{src} immunoprecipitates is shown in **Fig. 1**. The pp60 kinase band is clearly visible in the positive control virally transformed cell line (lane 4) but is not visible in the untransformed parent cell line (lane 2) or the normal rabbit serum control lanes (lanes 1 and 3). The antibody in the precipitate is also phosphorylated by the kinase but at a much lower level in the parental cell line. Unlabeled lysates of cells (500 µL) were immunoprecipitated as above using 3 µL of antibody or normal rabbit serum and 25 µL of Protein A beads; 2 µCi (4 µL) of [γ-^{32}P]ATP were then added to the immunoprecipitate, and it was left to phosphorylate for 30 min. After washing to remove excess ATP, the immunoprecipitates were solubilized by SDS and separated by PAGE in a 13% polyacrylamide gel.

3.1.4.2. FLUOROGRAPHY

This technique also utilizes the detection of photons/light emitted by scintillators to detect the presence of low levels of ^{35}S or ^{14}C ***(11)***. It can also be used to increase massively the detection limits of ^{3}H, a very weak α emitter. It is possible to measure as low an amount as 300 dpm of ^{3}H in a band in 24 h ***(12)***. The

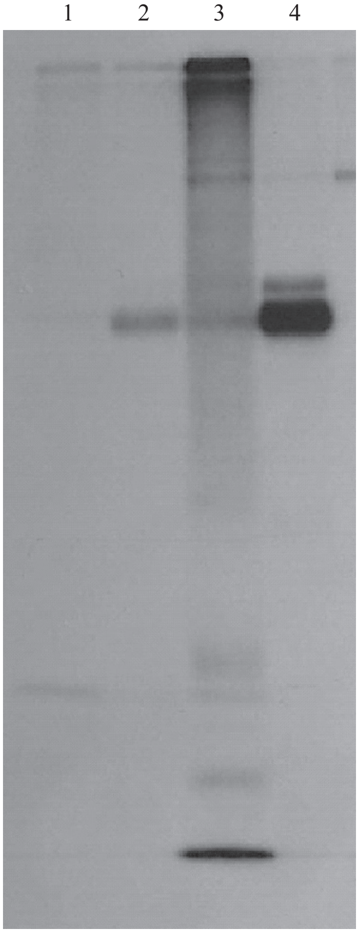


Fig. 1. A pp60src immunoprecipitate of rat fibroblasts (lanes1 and 2) and their virally transformed (A23) counterparts (lanes 3 and 4). Lanes 1 and 3 were control normal serum, lanes 2 and 4 were immunoprecipitated with pp60src antibody.

principles are exactly the same as those described for enhanced autoradiography. However, here the radioactive emissions are not strong enough to pass out of the dried gel and through the film to reach a scintillation screen. The scintillant has instead to be impregnated directly into the gel, as follows:

1. Totally dehydrate fixed gels by two 30-min to 1-h immersions in a 20X excess of dimethylsulfoxide (DMSO).
2. Immerse the gel in a saturated solution of the scintillator PPO (20% w/v) in DMSO for 1 h with gentle shaking.
3. Remove and place into a large excess of water where the scintillant immediately precipitates.
4. After 1 h, dry the gel normally and expose against preexposed X-ray film at –70°C.

3.2. Blotting

As mentioned in **Subheading 1**, blotting is described elsewhere in detail. Briefly, blotting is performed by the following steps:

1. Separate the protein mixture by SDS-PAGE and blot transversely onto a nitrocellulose or nylon membrane (*see* **Note 13**).
2. Block spare sites on the membrane (*see* **Note 14**).
3. Add the primary antibody (*see* **Note 15**).
4. After 1 h, wash the blot and add an enzyme-labeled anti-antibody (*see* **Note 16**).
5. Perform further washes and add a colored enzyme substrate. The substrate is precipitated onto the antigen by the enzyme ***(1,13)***. Dried blots (or photographs) can then be scanned for quantitation purposes.

3.2.1. Slot-Dot Blotting

To avoid the problems associated with denaturing the antigen you want to quantitate, it is possible to absorb a sample directly onto nitrocellulose by vacuum in either dots or slots. As either of the primary and secondary antibodies could be crossreacting with other components in the mixture and giving a false signal, it is essential to check that your antibodies are highly specific. This is carried out by using negative and positive control slots. These should contain a mixture of proteins that are known to be negative and a highly positive protein. Even then, artifactual results can occur. A more correct control experiment is to add varying amounts of your positive control in the presence of the same large amount of your negative control. The staining of the slots should then be in a linear relationship to the amount of positive sample added.

3.3. Precipitation and Blotting with Lectins

Precipitation with lectins is performed with microbatch lectin-affinity chromatography ***(14)***. This is used to take all the proteins with a given state of glycosylation out of a complex mixture prior to silver staining or blotting or ELISA quantitation. This could be used to study the glycosylated states of cellular antigens. Alternatively, it can be used to examine body fluids. I have used this procedure to study the fucosylation of serum glycoproteins in cancer progression ***(15–17)***, active and inactive arthritis ***(18)***, and inflammatory bowel disease ***(19)*** and have found similar changes in haptoglobin in the sera of “healthy” blood donors who smoke, compared with those found in cancer patients ***(20)***. This may be useful as a serum marker of risk of liver disease and/or cancer for the “healthy” population that smokes/drinks.

Figure 2 shows the serum glycoproteins precipitated from patients with five diseases by three lectins and demonstrates some of the major changes that can occur. When the fucose-specific *Lotus* lectin is used, cancer sera are characterized by strong bands around 43 kDa (lanes 2 and 3) and occasionally at

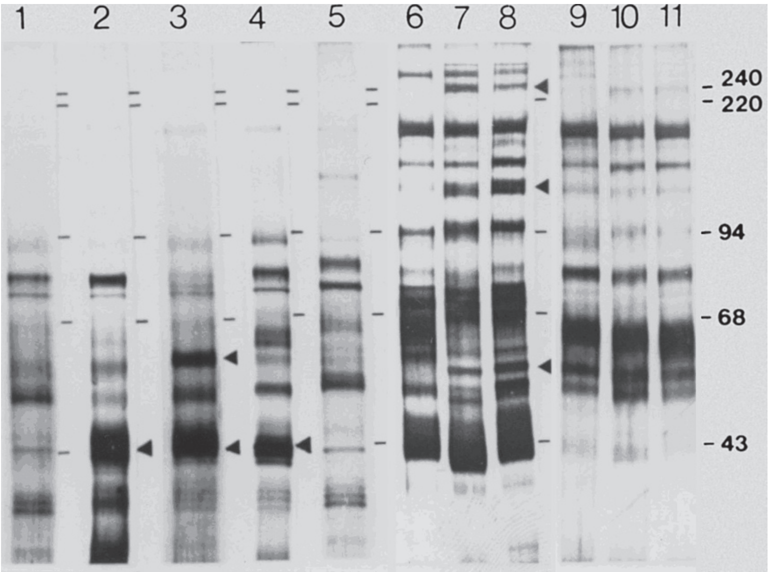
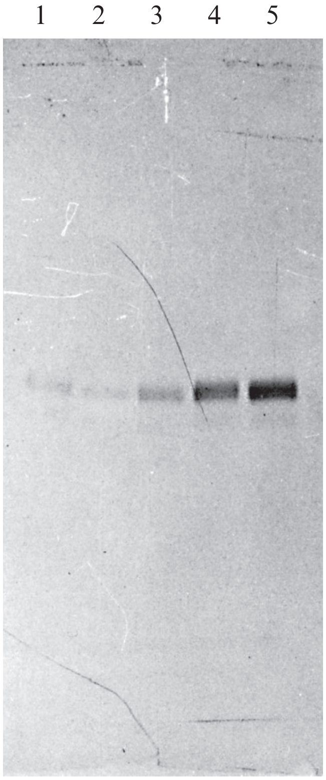


Fig. 2. Silver stained SDS-PAGE patterns of sera extracted with *Lotus* (lanes 1–5), WGA (lanes 6–8), or lentil (lanes 9–11) lectins. Lanes1, 6, and 9, healthy individuals; lane 2, advanced hepatoma, lane 3, recurrent cancer; lane 4, active rheumatoid arthritis; lane 5, bronchopneumonia; lanes 7 and 10, renal failure, lanes 8 and 11, liver cirrhosis.

57 kDa. These are haptoglobin and α_1-antitrypsin, the former being related to tumor burden ***(16)***and the latter to tumor progression ***(17)***. Active rheumatoid sera (lane 4) also contain abnormal haptoglobin, but this is of a lower molecular weight than that obtained in cancer sera ***(15)*** and is associated with high serum haptoglobin levels (4–6 g/L). Surprisingly, fucosylated haptoglobin is not detected in untreated broncopneumonia patients even when the total haptoglobin level is highly elevated (7–8 g/L). Changes could also be seen in these specimens when the N-acetylglucosamine-specific lectin wheat germ agglutinin (WGA) was used, but they were not as marked as those found with *Lotus* lectin. WGA extracts of renal failure (lane 7) and cirrhosis patients (lane 8) did give characteristically altered patterns that were highly reproducible. Here, however, very little change was found using lentil (lanes 9–11) or *Lotus* lectins. The altered proteins were identified by blotting as described above.

Figure 3 shows an α_1-antitrypsin blot of *Lotus* extracts from serial serum samples of a cancer patient that were collected at roughly 3-mo intervals. The fucosylated α_1-antitrypsin levels increased as her disease progressed. A weakly stained second band can also be seen just under the antitrypsin band, owing to the heavy chain human serum immunoglobulins crossreacting weakly with the

Fig. 3. An a1-antitrypsin western blot of fucosylated serum proteins extracted from a terminally ill cancer patient. Lanes 1–5 represented samples taken 0, 3, 6, 9, and 12 months after the commencement of treatment respectively.



AP-labeled sheep antirabbit second layer. A polyclonal rabbit antiserum (5 μL) was used at 1:1000 dilution followed by 5 μL of a second-layer AP-labeled sheep-anti-rabbit antiserum (1:2000).

Blotting with lectins requires the lectin to be coupled to another molecule such as digoxigenin. Your purified protein (or mixture of proteins) is slot or Western blotted, the blot is blocked (with nonionic detergent), and the lectin is added in the same manner as a first-layer antibody (*see* **Note 17**). After incubation with gentle shaking for up to 2 h, the blot is washed and an AP- or horseradish peroxidase-labeled antidigoxigenin antibody is added. Chemiluminescence can increase the sensitivity of this procedure and allow blots to be reprobed with different lectins ***(21)***. An alternative and now more commonly used procedure is to utilize biotinylated lectins. They are relatively easy to prepare, and many commercial antibiotin or AP-avidin second layers are available. They are often used to compare the glycosylation of recombinant proteins with their natural products and have also been used to study the expression of cell surface proteins in parasites ***(22)***.

4. Notes

1. $^{32}PO_4$ can also be used as a metabolic label for the specific labeling of phosphoproteins and measurement of kinase activity. However, here it is often more correct to label the cell lysates or even the specific kinase immunoprecipitates with [γ-^{32}P] ATP. This not only reduces the problems encountered because $^{32}PO_4$ also labels DNA and RNA but also minimizes the amount of ^{32}P required for protein labeling.
2. Do not label confluent cell cultures, as they will have a slower turnover rate than nonconfluent cultures. The cells may also run out of the labeling amino acid.
3. Low levels of dialyzed FCS (1–5%) are normally retained to maintain essential growth factors. Nonadherent cells are normally labeled at around 10^7 cells/mL.
4. Do not use trypsin to remove attached cells as you will alter cell surface protein expression. If labeled cells do not detach with EDTA, they should be washed *in situ* and then removed by rubbing them off the surface of the plate in the presence of lysis buffer.
5. It is essential to add nonradioactive KI; if this is not done, the radioactive ^{125}I sticks ionically to the surface of the cells and subsequently washes off in the washing steps. The addition of 0.5 μM "cold" KI, *before* the ^{125}I, saturates the cell surface, and all the ^{125}I is then available for labeling. Higher levels of "cold" KI reduce the efficiency of labeling.
6. If ^{32}PO4 is used as a metabolic label, it is essential to include large amounts of DNAase and RNAse and possibly micrococcal nucleases to digest small labeled pieces of DNA and RNA sticking to the proteins. Otherwise numerous artifactually phosphorylated proteins will be present in your mixture.
7. In both direct and indirect precipitation, the most critical factor is how the beads are washed after antibody–antigen complexes have been formed. If the beads are spun at any speed, the complexes split off the surface of the beads and are washed away. Furthermore, proteins stick nonspecifically to the newly revealed surface. I demonstrated this in 1982. Two identical immunoprecipitates were washed, one by gravity and one using a microfuge. A very clean precipitate with one band of the correct molecular weight was obtained with the beads that were allowed to settle out under gravity. However, numerous background bands with only a faintly visible correct band were obtained with the centrifuged immunoprecipitate.
8. Enough antibody has to be added to precipitate all the antigen present. Five microliters of polyclonal antisera, 5 µL of monoclonal ascites, or 50 µL of hybridoma tissue culture supernatant should easily be sufficient. The same amount of nonimmune sera or ascites should also be incubated with a second aliquot as a negative control.

9. In indirect precipitation, occasionally your antibody will not react very well with the protein A-Sepharose beads, and hence the antigen–antibody complex will not be precipitated properly. This is especially true with some subclasses of mice antibodies. It is possible to use alternative antibody binders with different binding specificities such as protein G-Sepharose. A simpler solution is to add 2 or 3 µL of a polyclonal sheep (or rabbit) anti-mouse antibody for a further 30 min after the initial antigen–antibody binding step ***(6,7)***. Sheep (and rabbit) antibodies bind very well to protein A and carry the mouse antibody-antigen complex onto the protein A. Rat monclonals may also be helped to immunoprecipitate with a sheep anti-rat second antibody.
10. If further chemical analysis of a purified immunoprecipitated antigen is required, the antigen can be released from the beads by the addition of a high (>9) or low (<4) pH buffer followed by centrifugation and immediate neutralization. Here direct immunoprecipitation is required or the purified antigen will be contaminated with the antibody ***(9)***. Trifluoroacetic acid (TFA; 0.1 *M*) instantly solubilizes purified antigens from antibody-coated beads without removing any antibody ***(9)*** from the beads.
11. The microcentrifugation step after addition of the SDS sample buffer can be omitted. The presence of a few microliters of Sepharose beads in a sample well does not have any deleterious effect on the migration of the immunoprecipitated proteins in discontinuous SDS-PAGE.
12. In both indirect autoradiography and fluorography, preexposed X-ray film is required to permit a linear detection of photons. It is very easy to do this. A flash gun is taken, and a red filter is taped over the flash. A strip of X-ray film is flashed from between 18 and 24 in. away (from above). This ensures a fairly even spread of the flash over the film. Normally the light is still far too strong. This can be further reduced by taping a piece of Whatman No. 1 filter paper over the filter followed by several layers of colored plastic tape. After each layer of tape is added, a strip of film is exposed and the film is developed until a background film OD of 0.15 is reached ***(12)***. X-ray film is two-sided; the side closest to the flash is placed against the scintillation screen or the impregnated gel.
13. There are two obvious major problems with Western blotting. The first is that a relatively high level of antigen is required in the sample, as most polyacrylamide gels have a maximum sample well size of 50 µL. Protein mixtures (serum samples, culture supernatants, and so on) are often preconcentrated by immunoprecipitation ***(9)*** or with lectins bound to Sepharose prior to blotting ***(14, 15)*** to circumvent this problem.
14. I always block remaining sites on the membrane with 0.5% Tween-20 after transfer. This can have the fortuitous effect of renaturing the antigen by displacing the SDS as well as blocking remaining active sites of the mem-

brane. I have also found that the alternative blocking reagents of 0.5% BSA or milk powder can cause very high backgrounds, especially when used in conjunction with nylon membranes, owing to nonspecific crossreactions with either or both the antibody preparations.

15. A primary antibody may sometimes crossreact with other proteins. More commonly it will not react at all due to the denatured nature of the antigen.
16. If an antibody has a high specificity but low avidity (as is the case with many antibodies to glycosylated antigens), the antibody/antibody–second antibody complexes will often fall off the blot during the washing steps, and no color will appear. This effect can be minimized or cured by two simple practical precautions:
    a. Use only an Fc-specific enzyme-labeled secondary antibody. This will then not interfere with the primary antigen–antibody reaction in the Fab region.
    b. The secondary anti-Fc antibody can be preincubated with the primary antibody for 1 h, and this primary-secondary complex can then be added in one step. Try to use anti-Fc-specific second-layer antibodies; this is especially important when blotting with first-layer monoclonals. Remember to preincubate the primary and secondary antibodies if the primary is suspected of having a low affinity. Not a lot else can be done about nonspecific crossreactions of either the primary or secondary antibodies. Samples should always be blotted in duplicate with one blot containing an irrelevant primary antibody raised in the same species as the correct primary. Specific staining is then guaranteed, especially if the staining is at the correct molecular weight.
17. Most if not all lectins require the presence of Ca^{2+} and Mg^{2+} ions. Most buffers for lectin immunoprecipitation ***(16)*** contain 5 m*M* Ca and Mg ions in isotonic Tris-HCl buffers (50 m*M*, pH 7.4). Blotting solutions are similar to those used with antibodies but always contain 1 m*M* Ca^{2+} and Mg^{2+}.

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Determination of Epitopes by Mass Spectrometry

Christine Hager-Braun and Kenneth B. Tomer

1. Introduction

According to the World Health Organization's AIDS Epidemic Update Report for December 2006 ***(1)***, somewhere between 34 to 47 million people worldwide are infected with the human immunodeficiency virus (HIV) and from 2.5 to 3.5 million people died from the disease in 2006. An epidemic like this makes it necessary to investigate the infection and its subsequent steps at the biochemical level in order to understand the process and to find means to prevent or interfere with an infection. As a response to an infection, the immune system develops antibodies that recognize regions, antigenic structures or epitopes, on proteins of the infectious agent. Determination of the epitopes provides invaluable knowledge for understanding the pathogenesis and developing of vaccines against HIV.

Mapping the epitope on the gp41 glycoprotein of the human immunodeficiency virus recognized by the human neutralizing monoclonal antibody 2F5 ***(2)*** illustrates an approach that is also applicable to other antigen/antibody complexes in characterizing immune responses or autoimmune diseases. Briefly, the antigen-recognizing primary antibody is covalently coupled to an immobilized Fc-specific secondary antibody. Subsequently, the antigen is bound to the primary antibody and proteolytically digested. The individual steps are monitored by matrix-assisted laser ionization mass spectrometry (MALDI/MS). The peptide that remains affinity-bound throughout the digestion steps reflects the epitope ***(2,3)***. A schematic outline of this approach is depicted in **Fig. 1**.

Although a variety of alternative techniques, such as peptide reactivity assays, mutation analyses, antigen proteolysis, crystallography, or nuclear magnetic resonance (NMR) spectroscopy, are available to determine epitopes ***(4–12)***, limitations to these approaches reduce the rate of success. Crystallography requires high concentrations of antigen and antibody and the formation of diffracting

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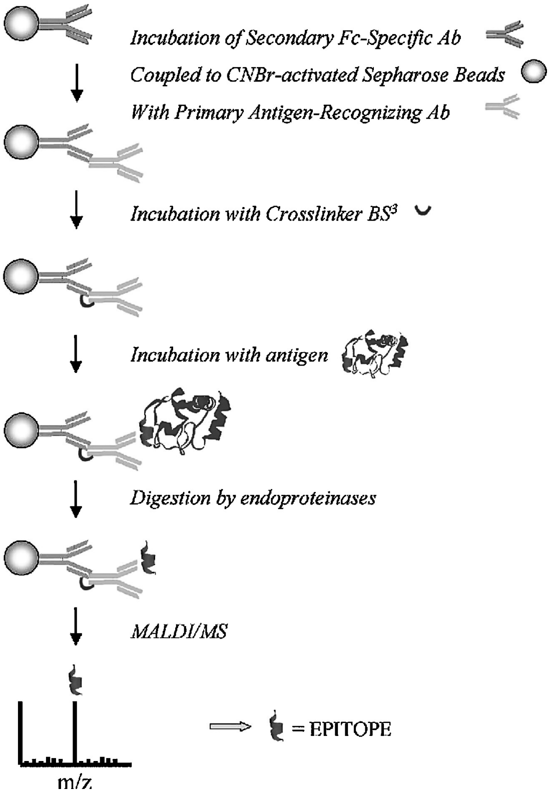
Incubation of Secondary Fc-Specific Ab
Coupled to CNBr-activated Sepharose Beads
With Primary Antigen-Recognizing Ab
Incubation with Crosslinker BS^3
Incubation with antigen
Digestion by endoproteinases
MALDI/MS
= EPITOPE
m/z

Fig. 1. Schematic outline of the epitope excision method followed by mass spectrometry. Ab, antibody; BS^3, *bis*(sulfosuccinimidly) suberate; CNBr, cyanogen bromide; MALDI/MS, matrix-assisted laser desorption/ionization mass spectrometry.

crystals. For NMR spectroscopy, the complex of antibody and antigen often exceeds the mass that can be analyzed. Peptide reactivity assays, either based on enzyme-linked immunosorbent assay (ELISA) or surface plasmon resonance imaging, are only applicable to linear epitopes but not to discontinuous epitopes or to antigens containing post-translational modifications. Mutation analyses focus only on specific amino acids by point mutations and are, therefore, tedious. Moreover, an altered binding affinity of the antigen to the antibody can be a false positive, when a distant mutation results in a conformational change of the antigenic structure. Other mass spectrometry-based approaches include epitope extraction ***(13–18)***, hydrogen/deuterium exchange ***(19,20)*** and differential surface modification ***(21–23)***. Epitope extraction is primarily useful for linear epitopes, but not for conformational or discontinuous epitopes. Differential surface modification and hydrogen/deuterium exchange are useful for discontinuous epitope characterization.

The method of enzymatic proteolysis of the antibody-bound antigen followed by MALDI/MS analysis as described here allows mapping of linear epitopes with three-dimensional conformation or post-translational modification with low sample consumption ***(2,24–34)***.

2. Materials

1. Cyanogen bromide (CNBr)-activated Sepharose 4B beads (Pharmacia Biotech, Piscataway, NJ).
2. Compact Reaction Columns (CRCs) with 35-µm column filters (USB, Cleveland, OH).
3. 1 m*M* HCl.
4. 0.1 *M* $NaHCO_3$, pH 8.2.
5. Coupling buffer: 0.1 *M* $NaHCO_3$, 150 m*M* NaCl, pH 8.2.
6. Anti-human Fc-specific IgG antibody (Sigma, St. Louis, MO) as secondary antibody.
7. Quenching buffer: 0.1 *M* Tris-HCl, pH 8.0.
8. 0.1 *M* sodium acetate, 0.5 *M* NaCl, pH 4.0.
9. Monoclonal antibody 2F5, IgG1 isotype, κ-chain (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) as the primary antibody.
10. Phosphate-buffered saline (PBS), PBS, pH 7.2: 100 m*M* Na-phosphate buffer, 150 m*M* NaCl, pH 7.2.
11. 10 m*M* *bis*(sulfosuccinimidyl)suberate (BS^3; Pierce, Rockford, IL) in PBS, pH 7.2.
12. SOSgp140 (JR-FL) glycoprotein, expressed as a gp120 glycoprotein covalently linked to the ectodomain of gp41 (gift from N. Schülke, Progenics Pharmaceuticals, Tarrytown, NY; ***16***).
13. Endoproteinase Lys-C (Wako, Dallas, TX).
14. 50 m*M* Tris-HCl, pH 8.0.
15. Carboxypeptidase Y (Roche Diagnostics, Indianapolis, IN).
16. PBS, pH 6.1: 100 m*M* Na-phosphate buffer, 150 m*M* NaCl, pH 6.1.
17. Aminopeptidase M (Roche Diagnostics).
18. Saturated solution of recrystallized α-cyano-4-hydroxycinnamic acid in ethanol/water/concentrated formic acid 45/45/10 (v/v/v).

3. Methods

3.1. Preparation of Immobilized Antibody Columns

1. Fill approx 0.2 g of dry CNBr-activated Sepharose 4B beads into a Falcon tube.
2. Mix the beads gently with 10 mL 1 m*M* HCl (*see* **Note 1**). Let the slurry equilibrate at room temperature for about 15 min, and then decant most of the supernatant.
3. Transfer 20 µL of beads slurry into each of two compact reaction columns (CRC).
4. Wash both CNBr-activated Sepharose columns with 6X 0.8 mL 1 m*M* HCl and 6X 0.4 mL 0.1 *M* $NaHCO_3$, pH 8.2.

5. To the drained beads in each CRC add 80 µL of 0.1 *M* $NaHCO_3$/150 m*M* NaCl, pH 8.2, and 20 µL of anti-human Fc-specific secondary antibody (48 µg) (*see* **Note 2**). Incubate at room temperature with slow rotation for 1.5 h (*see* **Note 3**).
6. Drain the beads and block any unreacted sites with 0.1 *M* Tris-HCl, pH 8.0, by rinsing the beads once with 0.4 mL and then incubating them in 0.4 mL for 1 h at room temperature with slow rotation.
7. Remove any antibody not covalently linked to the beads by a sequence of washing steps thereby alternating between 0.1 *M* sodium acetate/0.5 *M* NaCl, pH 4.0, and 0.1 *M* Tris-HCl, pH 8.0 with a volume of 0.4 mL each for three times. Finally, wash three times with 0.4 mL PBS.
8. Obtain a MALDI spectrum of the beads. Essentially, no ions should be detected at this point as the secondary antibody is covalently linked to the Sepharose beads (**Fig. 2A**).
9. Add 50 µL equivalent to 50 µg of the primary antibody 2F5 to one of the anti-human Fc-specific secondary antibodies containing CRC (= vial "Fc-specific Ab + 2F5"). To the other CRC, which will serve as the control (= vial "Fc-specific Ab"), add 50 µL PBS. Incubate both CRCs for 1 h at room temperature with slow rotation (*see* **Note 3**).
10. Drain the beads and save the drain in case the yield of captured primary antibody is low and a longer incubation time would be necessary.
11. Wash the beads three times with 0.4 mL PBS.
12. Check a 0.5 µL-aliquot of the beads by MALDI/MS to determine whether or not the primary antibody was successfully affinity-bound to the secondary antibody (**Fig. 2B**; *see* **Note 4**).
13. To stabilize the complex between the anti-human Fc-specific secondary antibody and the 2F5 primary antibody, the proteins are crosslinked. For this, add 10 µL of freshly prepared 10 m*M* BS^3 in PBS to vial "Fc-specific Ab + 2F5" and treat the control vial "Fc-specific Ab" the same way. Incubate both CRCs for 45 min at room temperature in the dark with slow rotation (*see* **Note 5**).
14. To quench the crosslinking reaction, drain the beads and wash twice with 100 µL 0.1 *M* Tris-HCl, pH 8.0.
15. Wash the beads three times with 0.4 mL PBS and obtain a spectrum of a 0.5 µL-aliquot of the beads by MALDI/MS. As the secondary and the primary antibody in the vial "Fc-specific Ab + 2F5" are now covalently linked, no ions from the antibodies should be observed in the spectrum (**Fig. 2C**).
16. From vial "Fc-specific Ab + 2F5," now crosslinked, transfer about one-fourth of the beads into a new CRC and add 50 µL of PBS. This vial will serve as the control for the proteolysis experiments.
17. Incubate the remaining beads of the vial "Fc-specific Ab + 2F5" with the antigen SOSgp140 using 50 µg of antigen solution (200 µL). Rotate slowly at room temperature for at least 2 h with up to overnight incubation if needed. In parallel, incubate the control beads without antigen.
18. Drain the beads and rinse three times with 0.4 mL PBS.
19. Obtain a MALDI/MS spectrum of a 0.5 µL-aliquot of the antigen-containing beads and of the control beads (**Fig. 2D**).

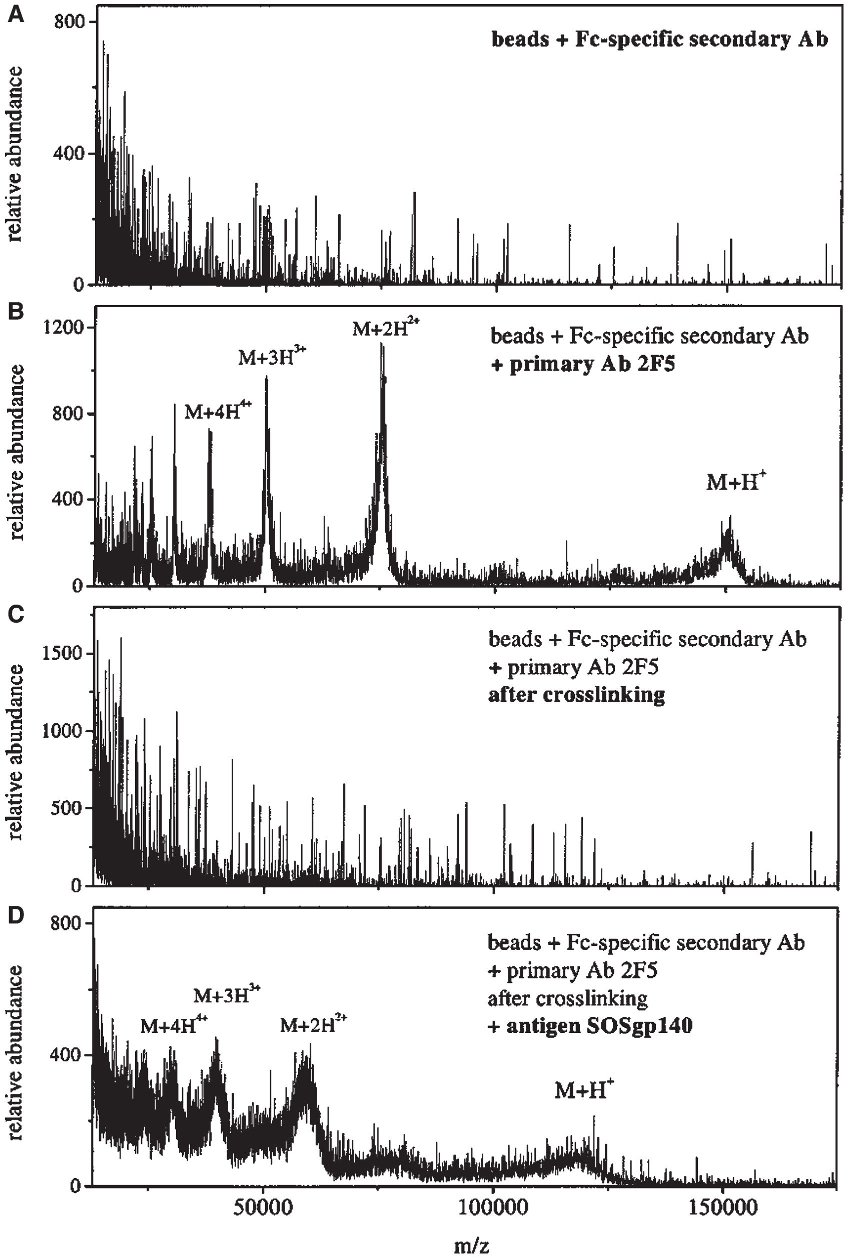

Fig. 2. MALDI/MS spectra of the Fc-specific secondary antibody (Ab) coupled to the CNBractivated Sepharose beads **(A)** followed by the addition of the primary antibody 2F5 before **(B)** and after **(C)** crosslinking with BS^3. **(D)** MALDI/MS spectrum of the affinity-bound antigen SOSgp140. The spectra were recorded in linear mode. (Adapted with permission from ***ref. 2***. Copyright 2001 American Society for Microbiology.)

3.2. Proteolysis of the Affinity-Bound Antigen SOSgp140

3.2.1. Endoproteinase LysC

1. Prepare a fresh solution of endoproteinase LysC (0.1 µg/µL) in 50 m*M* Tris-HCl, pH 8.0, and store it at 0°C (*see* **Note 6**).
2. Resuspend the beads containing the antigen as well as the control beads in 50 µL 50 m*M* Tris-HCl, pH 8.0 (*see* **Note 7**).
3. Add 50 µL of LysC solution (5 µg) to both CRCs.
4. Incubate both CRCs for 2.5 h at 37°C with slow rotation.
5. Wash the beads three times with 0.4 mL PBS.
6. Analyze a 0.5 µL-aliquot of both the antigen-containing beads and the control beads by MALDI/MS.

3.2.2. Carboxypeptidase Y

1. Prepare a stock solution of carboxypeptidase Y with a concentration of 0.5 µg/µL in deionized water just prior to use and store it on ice.
2. Resuspend the beads containing the antigen as well as the control beads in 50 µL PBS, pH 6.1.
3. Add 50 µL of carboxypeptidase Y solution to both CRCs.
4. Incubate both vials at 37°C with slow rotation.
5. After a 1-min incubation, drain the beads, wash them three times with PBS, pH 6.1, resuspend them in 50 µL PBS, pH 6.1, and remove a 0.5-µL aliquot of the beads for immediate MALDI/MS analysis.
6. Add fresh buffer (50 µL PBS, pH 6.1) and carboxypeptidase Y (50 µL) to the beads, continue incubation, and remove aliquots of washed beads at various time points like 1-min intervals (here: 2, 3, 4, 5, and 6 min) for MALDI/MS analyses. Although during the first 5 min a progress in digestion could be monitored (**Fig. 3**), at 6 min of incubation with carboxypeptidase Y, further digestion products were not detected (spectrum not shown). **Table 1** lists the ions observed in the MALDI/MS spectrum after 5 min of carboxypeptidase Y incubation and the corresponding amino acid sequences.

3.2.3. Aminopeptidase M

1. Wash beads containing the epitope and the control beads three times with 0.4 mL PBS, pH 7.2.
2. Resuspend the beads of both CRCs in 49 µL PBS, pH 7.2.
3. Add 1 µL of 5 µg/µL aminopeptidase M stock solution (as supplied by the manufacturer in ammonium sulfate solution) to both vials.
4. Incubate at 37°C with slow rotation.
5. At various time points like 1-h intervals (here: 1, 4, and 7 h), wash the beads with PBS and remove an aliquot for MALDI/MS analysis (**Fig. 4**). After the analysis, add new buffer and aminopeptidase to the beads and continue incubation. **Table 2** gives an overview of the ions found by MALDI/MS after 7 h of digestion and the assignments to the amino acid sequences.

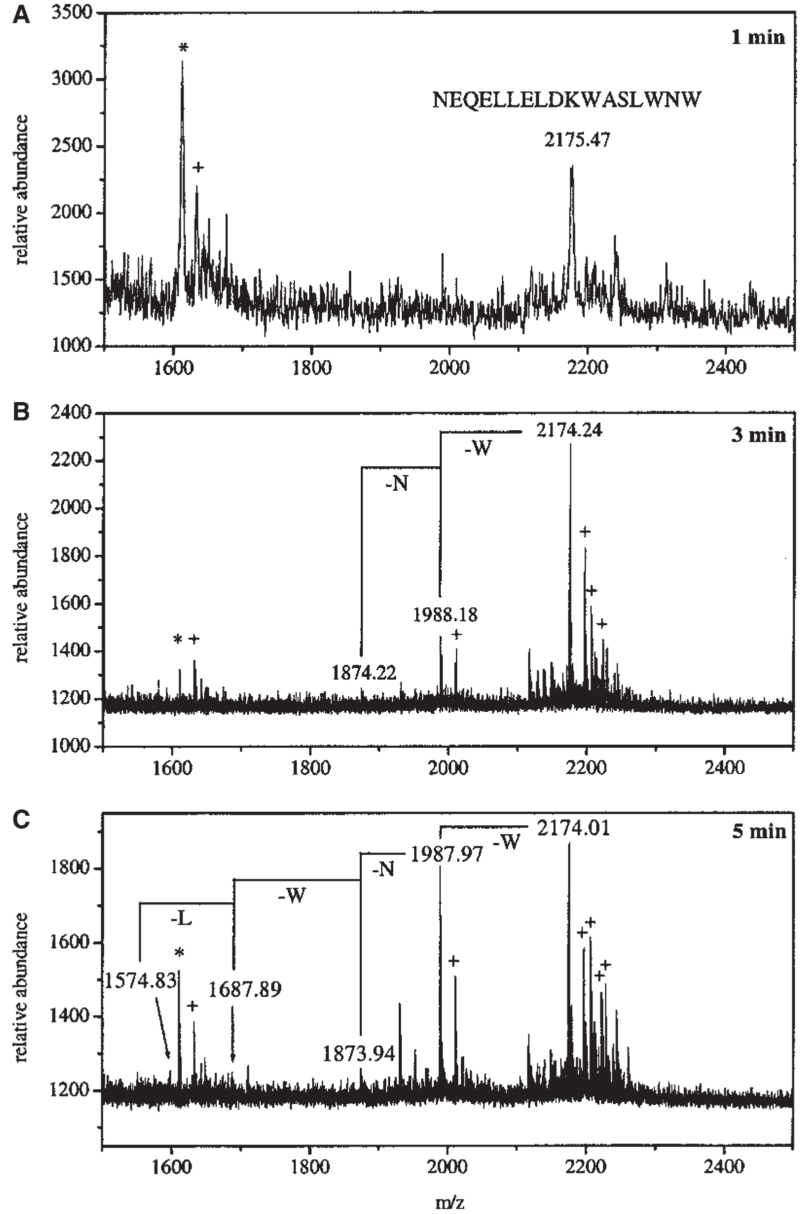

Fig. 3. MALDI/MS spectra obtained from the rinsed beads after digestion of affinity-bound SOSgp140 with endoproteinase LysC for 2.5 h followed by digestion with carboxypeptidase Y for 1 min (**A**), 3 min (**B**), and 5 min (**C**) to determine the fine structure of the epitope on the C-terminal end. Spectrum A was recorded in linear mode and spectra B and C in reflector mode of the MALDI instrument. The ion labeled with an asterisk represents a background ion, and ions labeled with a + represent Na^+-adducts and/or oxidized ions. (Adapted with permission from ***ref. 2***. Copyright 2001 American Society for Microbiology.)

Table 1
Products Found after Digestion of the Affinity-Bound SOSgp140 with LysC for 2.5 h Followed by Carboxypeptidase Y for 5 Min as Detected by MALDI/MS[a]

Amino acid sequence	Calculated M+H$^+$	Measured M+H$^+$
NEQELLELDKWASLWNW	2174.06	2174.01
NEQELLELDKWASLWN	1987.98	1987.97
NEQELLELDKWASLW	1873.94	1873.94
NEQELLELDKWASL	1687.86	1687.89
NEQELLELDKWAS	1574.77	1574.83

[a] *See* **Fig. 3C**.

Table 2
Products Found after Digestion of the Affinity-Bound SOSgp140 with LysC for 2.5 h Followed by Carboxypeptidase Y for 6 Min and Aminopeptidase M for 7 h as Detected by MALDI/MS[a]

Amino acid sequence	Calculated M+H$^+$	Measured M+H$^+$
NEQELLELDKWASLW*	1873.94	1874.17
NEQELLELDKWASLWN*	1987.98	1988.23
NEQELLELDKWASLWNW	2174.06	2174.30
EQELLELDKWASLWN	1873.94	1874.17
EQELLELDKWASLW	1759.89	1760.16

[a] *See* **Fig. 4B**. The amino acid sequences marked with an asterisk were already detected after the carboxypeptidase Y digest.

3.3. Mass Spectrometric Analysis

A 0.5-µL aliquot of the bead slurry or the drained liquid was spotted on a stainless-steel target and mixed with an equal volume of the saturated α-cyano-4-hydroxycinnamic acid solution. The target was left at room temperature to dry in air. MALDI mass spectra were recorded on a Voyager DE-STR (Applied Biosystems, Framingham, MA). Similar results should be obtained with similar equipment. External calibration of the mass range of interest was used.

3.4. Data Interpretation

1. Knowing the amino acid sequence of the antigen under investigation allows the researcher to determine which endoproteinase is best for digestion. A computer-based *in silico* digest gives a list of expected peptides with a molecular weight assigned. Choose the most suitable proteinase based on these *in silico* digest masses to allow MALDI/MS measurements with high mass accuracy on your specific instrument.
2. The amino acid sequence that contains the epitope should be protected by the antibody. Cleavage sites within the antigenic structure should not be accessible

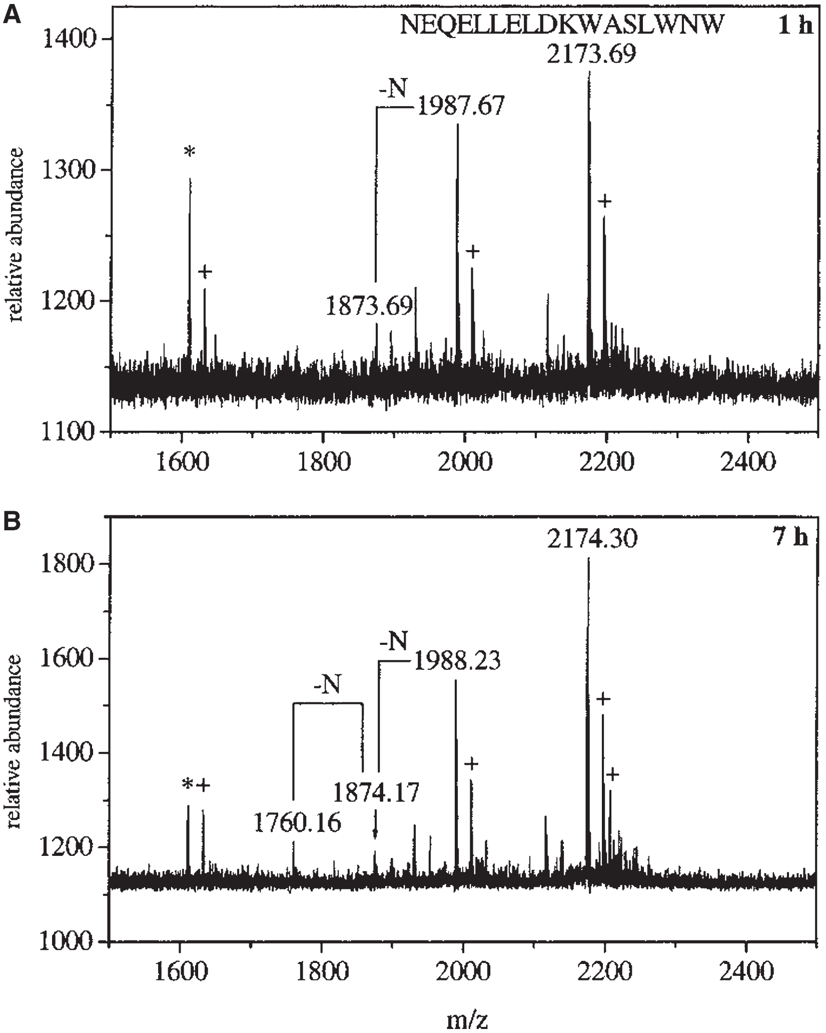

Fig. 4. MALDI/MS spectra obtained from the rinsed beads after digestion of affinity-bound SOSgp140 with endoproteinase LysC for 2.5 h followed by digestion with carboxypeptidase Y for 6 min and subsequent digestion with aminopeptidase M for 1 h (**A**) and 7 h (**B**) to determine the fine structure of the epitope on the N-terminal end. The spectra were recorded in reflector mode. The ion labeled with an asterisk represents a background ion, and ions labeled with a + represent Na$^+$-adducts and/or oxidized ions. (Adapted with permission from ***ref. 2***. Copyright 2001 American Society for Microbiology.)

to the endoproteinases, and, therefore, only adjacent cleavage sites will be used. To determine the fine structure of the epitope, digestion with carboxypeptidase Y will give information about the C-terminal end of the epitope, whereas digestion with aminopeptidase M will characterize the N-terminal side. The exoproteinases should only cleave "overhanging" ends of the affinity-bound peptide.

3. The ion detected in the MALDI spectrum after 2.5 h of LysC digestion and 1 min of carboxypeptidase Y digestion could be assigned to the amino acid sequence NEQELLELDKWASLWNW (**Fig. 3A**). Further incubation with carboxypeptidase showed the loss of W, N, W, and L from the C-terminus (**Fig. 3B** and **C**). The low abundances of the ions after the loss of N, W, and L, respectively, compared with the high abundance of the ion at *m/z* 1987.97 after the initial loss of W indicates that amino acids N, W, and L are partially protected by the antibody against proteolysis.
4. Using a heterogeneous sample after the carboxypeptidase Y digest for subsequent characterization of the N-terminus of the epitope results in a relatively complicated interpretation of the MALDI spectra. The ion at 1874.17 in **Fig. 4B** could be the result of the carboxypeptidase Y digest (NEQELLELDKWASLW) or could reflect the loss of the N-terminal asparagine residue from the ion at 1988.23 (NEQELLELDKWASLWN → EQELLELDKWASLWN) by the activity of the aminopeptidase M. The ion at 1760.16 indicates the removal of asparagine from the N-terminus of the peptide NEQELL ELDKWASLW with *m/z* = 1874.17 originating from the carboxypeptidase Y digest.
5. Based on the LysC digest, the carboxypeptidase Y digest, and the aminopeptidase M digest, the epitope recognized by the monoclonal antibody 2F5 is NEQELL ELDKWASLWN and is located near the C-terminus of gp41.

4. Notes

1. Handle the beads gently. Avoid any shaking, vortexing, or fast centrifugation, which might cause the beads to crush. All centrifugation steps to drain the beads are carried out in an Eppendorf (Westburg, NY) microcentrifuge at low force only (2–3 min at approx 80 *g*). For MALDI/MS analysis, only 10–20 beads are necessary to give sufficient results, although more may be used if required. Check under a microscope if enough beads were spotted for analysis.
2. The CNBr-activated Sepharose beads will react with primary amines such as the C-terminus of the heavy and light chains of the antibody as well as the ε-amines in lysine residues. Reactions are also possible with amines from buffers such as Tris, so in some cases it may be necessary to exchange the buffer of the antibody solution, for example, by gel filtration.
3. In some cases incubation times may need to be doubled.
4. In the MALDI spectrum, IgG molecules will give a singly charged ion at about 150 kDa. However, doubly and triply charged ions will be more abundant. Do not use dithiothreitol to reduce disulfide bridges as this will

cause the antibody to dissociate into its heavy and light chains. In case no ions can be detected in the MALDI spectrum of the beads, check the drain for presence of the antibody. If most of the antibody is detected in the drain, add the drain to the beads and continue to incubate at room temperature with slow rotation.

5. BS^3 [*bis*(sulfosuccinimidyl)suberate] is a homobifunctional *N*-hydroxysuccinimide ester that reacts with the α-amine at the N-terminus of a protein and the ε-amine of lysine residues. This crosslinker is water-soluble, and the storage of a stock solution is not recommended.
6. If using a different endoproteinase, a normally suggested protein/substrate ratio of 1:20 or higher is recommended. This ratio is higher than for in-solution digests but takes into consideration the possibility that the cleavage sites of the affinity-bound antigen in its native conformation are less accessible than in solution under denaturing conditions. Nevertheless, test the activity of the endoproteinase with the antigen in solution (or a readily available protein with similar molecular weight) to determine the rate of digestion.
7. In general you should wash the beads with the buffer that is recommended for the proteinase that you use. However, compatible components should be substituted for buffers and additives that are incompatible with subsequent MALDI/MS analysis. Avoid high concentrations of salt, as this can result in poor MALDI/MS signals, avoid glycerol, which will interfere with the formation of crystals for MALDI/MS analysis, and avoid the use of detergents because their presence will result in MALDI/MS ions with high abundance over a wide mass range, possibly suppressing the ions of the peptides of interest.

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Immunogen Preparation and Immunization Procedures for Rats and Mice

Mark Page and Robin Thorpe

1. Introduction

A high-titer antibody response usually requires use of an adjuvant for the first (priming) immunization. For most purposes, the immunogen is prepared by emulsification in a mineral oil containing heat-killed mycobacterium (Freund's complete adjuvant—FCA). The emulsion ensures that the antigen is released slowly into the animal's circulation, and the bacteria stimulate the animal's T-helper cell arm of the immune system. Further booster (secondary) immunizations are almost always necessary for production of high antibody levels, and these are given either in phosphate-buffered saline (PBS) or as an oil emulsion (bacteria are not normally included in the boosting injections; a suitable oil adjuvant is Freund's incomplete adjuvant—FIA). A large number of alternative adjuvants are available, but FCA/FIA (for priming and boosting respectively) usually produces maximal immune responses. However, FCA in particular can produce adverse effects in some cases and is not normally recommended for use in humans or primates Alum adjuvants are often chosen as an alternative and can be used in humans. Immunization with substances with molecular weights <3000 (such as peptides) are not normally immunogenic and will require conjugation to a carrier protein (*see* **ref. *1*** and Chapters 172–173), such as purified protein derivative (PPD) or keyhole limpet hemocyanin (KLH).

2. Materials

1. Freund's complete adjuvant (FCA).
2. Freund's incomplete adjuvant (FIA).
3. Phosphate-buffered saline (PBS).
4. Immunogen preparation.
5. 2-mL glass Luer lock syringes (two).

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6. Syringe coupler, Luer lock with female inlet and outlet ports (Sigma, St. Louis, MO).
7. Rat/mouse.

3. Methods

3.1. Immunogen Preparation (*see* Notes 1–5)

1. Dilute immunogen in physiological buffer (e.g., PBS without sodium azide) so that the preparation will contain 10–100 µg of protein in approx 300–600 µL/animal (Note: the final quantity will be 5–50 µg, since the preparation will be diluted with an equal volume of adjuvant). Mix the immunogen solution with an equal volume of FCA and draw up into a glass syringe or prepare directly in the syringe barrel.
2. Remove excess air from the syringe barrel, and connect to a second glass syringe with its plunger fully depressed via a double-hub connector.
3. Ensure the connections are tight and not leaking, and transfer the oil and immunogen solutions from one syringe to the other. Continue this action until the mixture is fully emulsified when it should appear as a creamy, white thick liquid.
4. Transfer emulsion into one of the glass syringes, remove from double-hub connector and empty syringe, and fit a small-diameter needle (the size of which will depend on the animal to be immunized and route of immunization).

3.2. Immunization Procedure

1. Prime mice or rats by immunizing with immunogen subcutaneously on the flanks and neck (0.1 mL/site, 3–5 sites). Do his by raising the skin between thumb and forefinger, and inserting the needle into the raised area at a shallow angle. A short narrow-diameter needle is preferred (0.4 × 27 mm) to avoid injection into the deeper body layers/cavities. The result should be a discrete lump under the skin.
2. Boost intraperitoneally after 14–28 d using the immunogen prepared in PBS via a short narrow-diameter needle. Administer the immunogen at one site in no more than 0.5 mL using the same total dose as that used for priming (usually 5–50 µg).
3. Three days after the booster immunization, withdraw blood from the tail vein with a needle (0.4 mm) and syringe, and use this as a positive control in screening assays during hybridoma production and as a check on the success of the immunization. Sacrifice mouse, and remove spleen aseptically.

4. Notes

1. Shake the complete adjuvant before use to disperse the Mycobacterium particles fully.
2. The emulsion is very difficult to recover completely from the walls of vessels, and so forth; therefore, it is inevitable that some will be lost during preparation. To minimize this, prepare the emulsion in glass syringes, one of which can be used for the immunization. If possible, prepare slightly

more than required to compensate for losses, which normally amount to around 10%.

3. If Luer connectors are not available, the emulsion can be prepared by vigorous shaking or mixing using a whirlimixer. This is less efficient at producing an emulsion and requires larger volumes of immunogen.
4. When using connected syringes, keep the hub connector as short as possible to avoid emulsion loss.
5. Use glass syringes to prepare the emulsion since the rubber seals of plastic syringes are not compatible with the mineral oil of the adjuvant.

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Making Hybridomas

Robert Burns

1. Introduction

In 1975 Kohler and Milstein reported *(1)* that immortal cell lines secreting antibody of a single specificity could be produced by the artificial fusion of splenocytes derived from immune mice and a tumour cell line derived from a murine myeloma. They called these cell lines hybridomas and the product from them monoclonal antibodies. The development of monoclonal antibodies opened up huge possibilities in all areas of antibody use as reagents could be created with specificity to a single epitope on the target protein. Additionally, antibodies could be generated to compounds that had previously been regarded as impossible when using conventional serum production. Monoclonal antibodies should however be seen as complementary to those derived from animal serum as each has its place in immunochemistry. The unique specificity, defined affinity and avidity of the monoclonal antibody are very desirable when looking at cell surface markers or single epitopes on a viral protein. In contrast, the broad specificity of polyclonal antibodies is a characteristic that may be desirable when screening for multiple strains of a virus or in techniques such as immunoaffinity purification.

The techniques for generating hybridoma cell lines have not changed much from the early work of Kohler and Milstein. Original work *(2)* used mice as donor animals and the mouse myeloma line NS-1 or its derivative NS-O (*see* **Note 1**) as the fusion partner. The murine system is probably still the most prevalent today although rats may be used for some antigens. Most murine hybridomas are produced from the physical fusion of spleen and tumour cells using low speed centrifugation and polyethylene glycol *(3)*. Recombinant hybridoma cells are selectively grown as only they have the necessary characteristics of immortality derived from myeloma cells, and a salvage pathway for purine nucleotide (Hypoxanthine-guanine

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phospho rybosyltransferase, HPRT) inherited from the splenocytes (*see* **Note 2**). Unfused spleen cells have a limited natural life span in tissue culture and the myeloma cell line lacks the purine nucleotide salvage pathway necessary for survival in the presence of the purine biosynthesis inhibitors (Aminopterin) contained in the selective medium (Hypoxanthine Aminopterin Thymidine, HAT).

Techniques such as electroporation and transfection have successfully been used for hybridoma production but are much less commonly used than fusion assisted by polyethylene glycol. Hybridomas may be unstable, as the techniques used to create them are fairly crude and may lead to a loss of cellular and genetic integrity. Instability is characterized by cell death after a few divisions or a loss or change in specificity. It is vitally important to test the hybridomas repeatedly to ensure that loss of specificity or ability to secrete has not occurred. The desirable qualities of monoclonal antibodies hinge on the fact that they are the products of a cell line derived from single parent cell. To ensure that this is the case it is important to aggressively clone the cells until a cloning efficiency of close to 100%, based on the quality of the antibody is achieved.

The vast majority of hybridomas generated in laboratories are destined to be discarded, as they will not have the desired qualities of antibody specificity, growth characteristics or cloning ability required. In most cases, it is more practical to derive a new cell line rather than try to continue with one that is less than ideal. It is very important to have in mind the qualities of the cell line that are required along with the characteristics of the antibody that are needed prior to embarking on hybridoma production.

Other species hybridomas, including human have been produced but are generally created by the use of viruses conferring cellular immortality. Artificial immunization of the donor is often not practical or ethical and so cell lines are often derived from peripheral lymphocytes obtained from individuals naturally immune to the target substance. Some human monoclonal antibody secreting cell lines have been derived from spontaneously occurring myelomas but this line of approach is frequently unrewarding as the probability that the antibody will be one of interest is remote.

Nonsecretory myeloma fusion partners with defective purine nucleotide biosynthesis pathways do now exist for a number of species including humans and so hybridoma production by cell fusion using PEG is now a possibility.

Hybridoma production can be broken down into four processes, immunization of donor animals, cell fusion, cell selection and expansion and each of these stages is important for the quality of the final product. Antigens used to immunize animals must be representative of the target substance (*see* **Note 3**) or the likelihood of producing cell lines with the correct specificity is remote. Cell fusions must generate hybridomas but steps must be taken to ensure that neither too few nor too many are generated. Cell lines must be selected using strict criteria to ensure that desirable qualities in both cell growth and stability are present along with the specificity, avidity and affinity required for the final testing format. It is always advisable to screen primary cell lines using the assay format envisaged for the final test (*see* **Note 4**). Monoclonal antibodies may perform perfectly well in one assay format but may not for one reason or another convert to another.

2. Materials

1. 4 female Balb-c mice (*see* **Note 5**).
2. NS-O myeloma cell line.
3. Basal RPMI 1640 medium requires supplementation with sodium pyruvate, L-glutamine and Penicillin/Streptomycin before use. The supplements are supplied as concentrates of X50 or X100 and the appropriate amounts should be added. Standard tissue culture media for hybridoma production contain 5%, 10%, or 15% FBS (*see* **Note 6**). Sufficient quantities should be prepared in advance and a sterility check should be performed on them prior to use.

   All prepared media should be stored at −20°C until required and used within 28 days of thawing. L-Glutamine has an effective lifespan of about 28 days in prepared medium and will degrade giving rise to ammonia after this time.
4. HAT medium: Add 5 mls HAT supplement to 500 ml RPMI 1640 medium containing 15% FBS. Check sterility prior to use. Store at −20°C.
5. Allogeneic mixed thymocyte medium (MTM) is a conditioned medium used to aid cells recover from the fusion process. It also encourages division of hybridoma cells following dilution cloning when colonies derived from single cells are required. Rat thymi are used for this purpose to minimize the number of animals required to produce adequate numbers of cells. The medium contains a number of "helper" cytokines produced by the thymocytes. It is important that the thymocytes used are derived from two different rat strains as this causes co-stimulation and enhanced cytokine secretion by the cells. MTM must always be diluted with RPMI 1640 medium containing 15% FBS and is usually used at a dilution of 10%–15%. A number of proprietary supplements are available which can be used in the place of MTM. All of these contain cytokines derived from a variety of sources which provide the necessary stimulation for hybridoma cell division.
6. Allogeneic mixed thymocyte conditioned medium: Obtain two 6-week-old rats of two different strains (e.g., Sprague and Wistar). Kill rats humanely and remove the thymus glands aseptically. Homogenize the thymus glands using the frosted ends of glass microscope slides (see method for spleen homogenization) and suspend in 10 ml PBS. Wash by centrifugation 400 g and resuspend in 10 ml PBS. Add the thymocyte suspension to 1 litre RPMI 1640 medium containing 15% FBS and distribute into 4 X 225 cm T flasks. Incubate the cells for 30 to 40 h 37C/5% CO_2 (it is important that incubation is not longer than 40 h or undesirable cytokines will be produced). Harvest the conditioned medium is by centrifugation 500 g. The medium should be tested for sterility and stored at −20°C in 20 ml aliquots.

7. Freezing medium: Add 50 ml DMSO to 500 ml FBS Dispense in to 20 ml aliquots. Test sterility. Store at −20°C.
8. PEG medium for cell fusions (*see* **Note 7**): Autoclave 20 g PEG 4000 MW in a glass universal 121C/24 min. When cool enough to handle quickly add 20 ml RPMI 1640 without FBS. Mix well and dispense in 2 ml aliquots. Check sterility. Store RT in dark (*see* **Note 6**).
9. 96 well tissue culture plates.
10. 24 well tissue culture plates.
11. 25 cm "T" tissue culture flasks.
12. Freezing medium.
13. Cryovials.
14. Sterile glass petri dish and one pair of sterile "frosted end" glass microscope slides.

3. Methods

3.1. Immunizations

Mice should be obtained from a reputable source and should have be maintained under standard condition as required by local animal welfare legislation. Female mice can be housed together and it is usual to indelibly mark individuals by tattoo, ear punch, or electronic chip, so that the immune response for each individual animal can be monitored.

1. Prepare antigens at approximately 1 mg/ml in physiological saline.
2. Inject the mice intraperitoneally with 0.1 µl of antigen mixed with an appropriate adjuvant on days 0, 14, 28 and 44. Take a test bleed on day 51 and assess the serum for the presence of circulating antibody to the antigen.
3. If the immune response is good (*see* **Note 8**) rest the animals for a minimum of 8–10 weeks prior to cell fusion work. If the immune response is poor give another immunization on day 61 and assess the circulating antibody level on day 68.
4. Prior to carrying out a fusion, give the mouse with the highest antibody titre a booster dose of 50–100 µl antigen *without* adjuvant intraperitoneally. Three days later kill the mouse by cervical dislocation and remove spleen aseptically.
5. If all mice have responded well to the immunizations give all of them a booster dose, harvest the spleens, and cryogenically store the splenocytes for use at a later date (*see* **Note 9**). Store the spleen cells cryogenically as described in 3.6.

3.2. Preparation of the Myeloma Cells

NS-O myeloma cells are normally kept stored cryogenically and so should be resuscitated 3 days prior to carrying out the fusion.

1. Quickly thaw a frozen aliquot of NS-O cells either in a 37°C water bath or between the palms of the hands. Once the pellet has melted add 1 ml 37°C RPMI 1640 medium supplemented with 10% FBS and draw up into a Pasteur pipette. Transfer

the cells to a 225 cm "T" flask and add 50 mls RPMI 1640 medium containing 5% FBS and place in a tissue culture incubator 37°C/5% CO_2.

2. Inspect the cells on day 2, they should be semiconfluent and adherent to the flask base. Increase the volume of RPMI 1640/5% FBS medium to 75 ml and return to the incubator.
3. Inspect the cells on day three, they should be almost fully confluent on the flask base. Discard the medium, add 10 ml cold PBS containing 0.02% EDTA and leave for a few min to allow the cells to detach. Split the cells equally between 2 flasks and add 75 ml RPMI 1640 medium/5% FBS to each flask and return to the incubator.
4. On day four remove the medium from each flask and replaced with 20 ml cold PBS containing 0.02% EDTA. After a few min the cells will detach from the flask surface. Harvest the cells by gently tapping the flask and pouring the cell suspension in to sterile universal containers. Wash the cells by centrifugation 300 g and resuspend in 10 ml cold PBS.

3.3. Preparation of Splenocytes

Keep the spleen on ice, in RPMI 1640 medium without FBS until the splenocytes are harvested. Spleens can kept in this way for 1–2 h without significant loss of splenocyte viability.

1. Decant the spleen in to the sterile glass petri dish and gently dissociated by rubbing between the frosted ends of the glass microscope slides. It is sometimes necessary to break up the spleen a little prior to dissociating using either the ends of the microscope slides or a pair of dissecting scissors. After dissociation there will be fibrous tissue remaining from the spleen capsule and a red liquid containing the splenocytes.
2. Aspirate the red liquid from the fibrous tissue and place into a sterile universal container. Allow the contents of the universal container to settle for a few min then aspirate or "pour off" the supernatant from the residual tissue fragments (*see* **Note 10**).
3. Wash the splenocytes with PBS by centrifugation at 400 g and resuspend the pellet in 10 mls of cold PBS.
4. Remove 2.5 mls of splenocyte suspension for cell fusion.
5. Harvest the remaining cells by centrifugation, resuspend in freezing medium (2 ml spleen) and dispense into cryotubes in 0.5 ml aliquots. Freeze the cells and store cryogenically.

3.4. Cell Fusion

1. Mix the washed NS-0 cells harvested from the 2 T flasks with one quarter of the splenocytes from one spleen.
2. Pellet cells by centrifugation 400 g 5 min.
3. Discard supernatant and slowly add 1 ml PEG/RPMI mixture.
4. Gently resuspend the pellet by swirling.

5. Pellet cells by centrifugation 250 g 5 min.
6. Slowly overlay cell/peg layer with 5 mls RPMI 1640 with no FBS and then gently swirl to create a cell suspension.
7. Pellet cells by centrifugation 400 g 5 min.
8. Discard PEG/RPMI mixture and replace with 5 mls HAT medium, do not disturb the cell pellet then incubate the cell pellet for 5–10 min at 37°C.
9. Resuspend the cells by swirling and add 7.5 mls HAT and 7.5 mls Allogeneic Mixed Thymocyte Medium.
10. Dispense cells suspension into 96 well tissue culture plates (0.2 ml/well) (*see* **Note 11**).
11. Place in 37°C/5% CO_2 incubator.
12. After 7 days examine the wells for the presence of hybridomas and given an additional 0.1 ml HAT medium.
13. Test wells exhibiting growth for the presence of monoclonal antibodies when the hybridoma growth covers approximately 25% of the base of the cell well. The assay system used should mimic the final test format required. Commonly, TAS ELISA is use for screening hybridomas for specific antibody. It is important when testing hybridomas that a suitable negative is included as background antibody activity from unfused spleen cells may give apparently positive results. Up to 200 µl of medium can be harvested from each cell well, which can be split to assay against specific antigen and a suitable negative.

3.5. Stabilizing Hybridomas

Wells testing positive for specific antibody should be assayed more than once to ensure that activity continues over the course of a few days. The health of the cells should be checked daily to ensure that overcrowding does not occur and once the cells are confluent in the cell well they should be subcultured into 24 well plates. The cells must also be cloned by limiting dilution at this stage and plated out at 1 cell/well. It is useful to test the isotype of the secreted antibody when the cells are growing in the 24 well plates. This can be accomplished using a number of “dip stick” assays giving results in a matter of a few min. The isotype of the antibody is important for development of the antibody if purification and reagent development will be required. Antibodies of the sub classes G1, G2a, and G2b are the most desirable for further development as they are readily purified and are relatively stable. Antibodies with the G3 isotype, most commonly produced from bacterial antigens may be inherently unstable and liable to spontaneous aggregation.

Approximately 50% of hybridomas will secret antibody of the M subclass. IgM is a large molecule that does not readily purify but may be used for TAS ELISA. Some antigens will only give rise to IgM secreting hybridomas and are know as anamnestic antigens. These substances are frequently highly glycosylated and do not invoke a “memory” response in the immune system.

The result of this is that B-lymphocytes never mature beyond the production of IgM regardless of the number of immunizations the animal is given.

3.5.1. Limiting Dilution Cloning

1. Agitate the contents of the cell well using a Pasteur pipette and then using a pipette with sterile tip draw up 2X 10 ul aliquots of cells. Add one aliquot to 1 ml warm RPMI 1640 medium with 15% FBS. Add the other aliquot to 10 µl of Trypan blue dye and count the cells using a Neubauer counting chamber.
2. Calculate the cell count in the 1 ml tube using the formula: (total cell count/number of grid squares counted) × 10^4 × 2 = cells/ml.
3. Calculate the volume of cell suspension from the 1 ml tube that would contain 60 cells.
4. Transfer the appropriate volume of the medium and cells from the 1 ml tube to the 9 ml cloning medium and dispense 150 µl/well in 60 wells of the cloning plate.
5. Place the cloning plate into the tissue culture incubator 37°C/5% CO_2 for one week and then inspect for growth. Most cell wells should have single hybridoma colonies growing in them. **It is important at this stage to record the wells containing single colonies**. Give each of the cell wells 50–100 µl of cloning medium and reincubate until cella are between 30–50% confluent.
6. Test the wells which originally contained single colonies for specific activity; and record the numbers which are positive.
7. Subclone the cells using the method above until 100% of the clones are positive for the specific antibody. Occasionally some cell lines will not achieve a cloning efficiency of 100% regardless of the number of subcloning attempts. In these cases a final clone should be selected, rapidly multiplied and cryogenically stored. Cells from these stores should never be used to produce additional cell stocks without subcloning again.
8. Choose one of the clones from the final cloning as the cell line, expand it in tissue culture and cryogenically preserve stocks of it as the master cell bank (at least 12 vials).

3.6. Preserving Cells Cryogenically (Freezing)

1. Keep flasks of cells in log phase of growth by subdividing when almost confluent.
2. Make sure that caps are tightened on flasks and then sharply tap to dislodge cells.
3. Pour off cell suspension into a sterile plastic universal container.
4. Pellet cells at 400 g for 5 min.
5. Pour off medium, add 200 µl 1% Sodium azide solution and retain for assessment of antibody activity.
6. Tap the cell pellet and add 0.5 ml **cold** freezing medium.
7. Aspirate with Pasteur pipette to resuspend pellet then transfer to a 2 ml cryogenic vial.

8. Cells must be frozen at approximately 1°C/min and this can be achieved by putting the cryovials into an expanded polystyrene container with a wall thickness of 0.5 cm and then placing them in a −70°C freezer.
9. Transfer the cells to liquid nitrogen storage within 3 days of freezing at −70°C, they will remain viable for many years.

4. Notes

1. It is preferable to use NS-0 as a fusion partner, it does not secrete a constitutive antibody but has the ability to produce monoclonal antibodies once fused to an immune spleen cell. NS-1 has a constitutive antibody which may cause interference in screening assays for recombinant hybridoma cells.
2. Cell lines are available which have other defective pathways, aminopterin cannot be used to select for their growth and an alternative agent must be used.
3. Antigens must be representative but do not need to be identical to the target substance. Synthetic peptides modelling single epitopes and fusion proteins containing homologous regions to the target can be used very effectively as antigens.
4. It is not always possible to use exactly the same format for screening as will be used in the final assay but it is advisable to attempt to ensure that the "position" that the Mab will occupy in the final format is the position that is used for screening. For example mabs which will eventually be conjugated to enzymes and used in DAS ELISA should be screened for using TAS ELISA. This ensures that the epitope-binding portion of the antibody is in the same position as it will be in the final test format with the analyte bound to the coating antibody.
5. Balb-c mice are normally used for mab production, partly because they are the strain of mouse that the NS-0 cell was derived from and also because they are easily handled and the females can be communally housed.
6. Batches of FBS vary enormously in their ability to support cell growth and it is important to establish that the batch you are buying will be suitable. Most suppliers will allow you to reserve quantities from a batch and will provide small samples for testing. Usually a few batches are tested at the same time and the best performer then selected. Testing is carried out by cloning an established hybridoma line by limiting dilution in medium containing the test FBS and then observing numbers of resulting colonies.
7. Polyethylene glycol 4000 MW is normally use for cell fusions. Batches may vary in their ability to produce viable hybridomas and it is wise to test a few batches from different sources prior to undertaking hybridoma project work. A number of biochemical suppliers now produce ready-to-

use PEG/media solutions in sterile ampoules, which workers may find is a more practical source.

8. If the antigen to be used is derived from fungal mycelium, bacterial cell walls or is known to be highly glycosylated then repeated immunization is of no value. Antigens of this type are known to be anamnestic and do not produce "memory" B-lymphocytes. The animal sees each immunization as a primary challenge and the likelihood of producing an IgG response is remote.
9. Frozen spleen cells work well for cell fusions and this approach allows a greater degree of flexibility in performing fusions than using splenocytes directly *ex vivo*. The vial of spleen cells should be rapidly thawed in a 37°C water batch or between the palms of the hands. 1 ml of PBS is then added to the vial and the contents aspirated and added to 5 ml PBS. The cells are washed once by centrifugation 400 g and resuspended in 5 ml cold PBS; they should then be stored on ice until required. Spleen cells that have been frozen may have a tendency to clump and the pellet will look very pale. This does not affect their ability to produce viable hybridomas.
10. Disposable cell strainers that fit into the top of universal containers may be used to remove large fragments of spleen tissue.
11. The volumes of media used during cell fusion and subsequent plating in to tissue culture wells do not need to be accurately measured. Disposable Pasteur pipettes can be used to approximate volumes and most manufacturers publish specifications including drop volume for dispensing cells and medium.

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Growing Hybridomas

Gary Entrican, Catherine Jepson, and David Deane

1. Introduction

The fusion of antigen-primed B cells with transformed myeloma cells results in immortalized hybridomas that secrete antibodies. The subsequent cloning of the hybridomas gives rise to cell lines secreting monoclonal antibodies (MAbs) of a single specificity ***(1)***. This technology has had a tremendous impact on research and medicine, with MAbs being used to identify and characterize the biological significance of myriads of molecules. The outcome has been the development of diagnostic tests and therapies for the detection and management of disease ***(2)***. The amount and purity of a MAb required for any given purpose can vary greatly. Here we describe the procedures involved in the maintenance and management of hybridomas and suggest techniques for maximising yields.

2. Materials

1. RPMI 1640 1× liquid without L-glutamine, without HEPES, stored at 4°C (Gibco Invitrogen Corporation, Inchinnan Business Park, Paisley, UK).
2. 100 mM L-glutamine solution, stored at −20°C (Sigma, Poole, Dorset, UK).
3. 1 *M* HEPES solution, stored at 4°C (Sigma).
4. Nutrient broth No. 2 (NB): 25 g dissolved in 1 liter of sterile H_2O, stored at 4°C (Oxoid Ltd, Basingstoke, Hampshire, UK).
5. Oxaloacetate, pyruvate insulin (OPI) media supplement solution: 1 vial dissolved in 10 ml sterile H_2O, stored at −20°C (Sigma).
6. Penicillin/streptomycin solution, stored at −20°C: 10,000 International Units/ml benzypenicillin sodium BP (Britannia Pharmaceuticals, Surrey, UK) and 10,000 μg/ml streptomycin sulphate (Gibco Invitrogen Corporation).
7. Fetal bovine serum (FBS), heat-inactivated at 56°C for 30 min to destroy complement and stored at −20°C (Gibco Invitrogen Corporation).

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8. Costar/Corning 96- and 48-well tissue culture plates (Corning Incorporated, New York).
9. Corning 25 cm^2, 75 cm^2, 225 cm^2 vented sterile tissue culture flasks (Corning Incorporated).
10. Costar/Corning 5 ml, 10 ml, 25 ml disposable sterile pipettes (Corning Incorporated).
11. MiniPERM classic kit, molecular weight cutoff 12.5 KDa. Kit includes twelve miniPERM bioreactors (Greiner Bio-One Ltd, Gloucestershire, UK).
12. CellPROTECT, anti-shearing agent for high-density culture (Greiner Bio-One Ltd).
13. AntiFOAMa (Greiner Bio-One).
14. Universal turning device, holds four miniPERM bioreactors (Greiner Bio-One Ltd).
15. Disposable hypodermic syringe, 50 ml Luer (Greiner Bio-One Ltd).
16. Disposable hypodermic syringe, 2 ml Luer (Greiner Bio-One Ltd).
17. Spare Luer-Lock screw caps for production modules. Six caps per case, autoclavable (Greiner Bio-One Ltd).
18. Spare filter screw cap for nutrient module: thirty caps per case, autoclavable (Greiner Bio-One Ltd).
19. MiniPERM stand, for mounting the miniPERM bioreactor during inoculation, sampling and cell harvest: Four stands per case (Greiner Bio-One Ltd).
20. Neubauer haemocytometer (Fisher Scientific UK, Bishop Meadow Road, Loughborough, Leicestershire, UK).
21. Cryopreservation tubes (Nunc A/S, DK-4000 Roskilde, Denmark).
22. DMSO Hybrimax (Sigma).
23. ‘Mr Frosty’ cryopreservation 1°C freezing container (Nalgene, Nalge Europe Ltd, Thorn Business Park, Hereford, UK).
24. 2-propanol (Rathburn Chemicals, Walkerburn, Scotland, UK).
25. Nigrosin, dissolved to a final concentration of 0.1% w/v in PBS containing 2% FBS, filter-sterilized and stored at 4°C (Sigma).
26. RPMI is used as the standard base medium for culturing hybridoma cell lines. The following supplements are added to each 500 ml bottle of RPMI: 10 ml penicillin/streptomycin (*see* **Note 1**), 10 ml glutamine (*see* **Note 2**), 5 ml HEPES and 50 ml FBS (*see* **Note 3**). The contents should be mixed by swirling, not by inversion, thereby avoiding leakage and contamination. Hereafter this mixture is referred to as complete medium (CM). Two × 1 ml aliquots of CM should be removed and added to 2 × 5 ml aliquots of nutrient broth (NB) for sterility testing. Keep one NB at room temperature for 3 days and incubate the other at 37°C for 3 days. Check the NBs for evidence of microbial contamination prior to using the CM for the first time. Store CM at 4°C and use within 2 weeks of preparation (*see* **Note 2**).

3. Methods

The methods in the following sections describe (1) the growth of cloned hybridoma cell lines, (2) high-density culture systems required for bulk production of MAbs, and (3) the generation of validated cell banks where hybridomas can be stored for future retrieval. All procedures that involve manipulation of cell lines are conducted within the confines of a Class II microbiological safety cabinet using sterile equipment. Cell cultures are maintained at 37°C in an atmosphere of 5% CO_2 in air and 100% humidity. It is preferable to use tissue culture flasks that have filter-vented caps to minimize the chances of contamination. Several hybridoma cell lines were used in the following procedures: 36F ***(3)***; VPM 20, VPM 21, VPM 22 ***(4)***; 73B ***(5)***; 3C2 and 8D8 ***(6)***. These cell lines have individual characteristics, but the procedures described here are generic in nature and can be applied to all hybridoma cell lines, taking into account the comments under **Subheading 4**.

3.1. Expansion of Cloned Hybridomas

1. Transfer hybridoma cells from the 96-well cloning culture plate to a 48-well culture plate in 0.4 ml fresh CM (*see* **Note 4**), using gentle pipetting to dislodge the cells from the plastic (*see* **Note 5**).
2. Two days later transfer the cells to a 25 cm^2 flask in 2 ml fresh CM (*see* **Note 6**). Next day, add 3 ml CM to feed the cells.
3. The following day, gently pipette the 5 ml of medium in the flask several times several to dislodge the cells and transfer the total volume to a 75 cm^2 flask. Add a further 5 ml fresh CM immediately to the flask to feed the cells. Add 10 ml fresh CM 24 h later.
4. The next day transfer the 20 ml containing the cells to a 225 cm^2 flask using gentle pipetting as before, then 30 ml fresh CM is added immediately.
5. The following day dislodge the cells from the flask by pipetting and add 10 ml of the resulting suspension to each of five 225 cm^2 flasks. Add 40 ml fresh CM per flask. This protocol of passaging 10 ml of cell suspension at a 1:5 ratio can be followed for expansion of hybridomas either for generating cells for high density cultures (***3.2***) or for preparation of cell banks (***3.3***). Check the cells for *Mycoplasma* contamination (*see* **Note 7**) and analyse the supernatant for antibody production (*see* **Note 8**) before progressing to either of the next stages.

3.2. High Density Culture

There are multiple means by which high density hybridoma cultures can be achieved. The objective is to maintain cloned hybridomas in a healthy condition in the lowest possible volume of CM to maximize antibody production and facilitate downstream antibody purification. There are various products on the market such as multi-layered tissue culture flasks, dual-chamber flasks

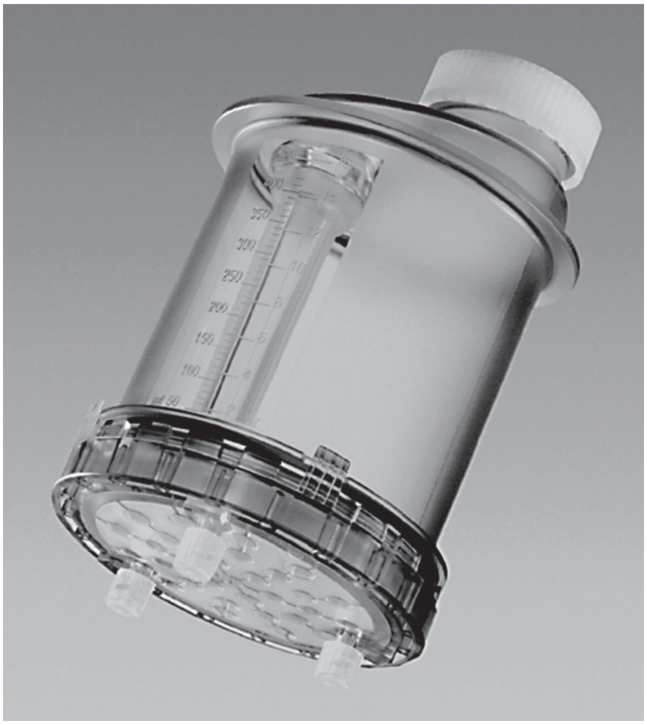

Fig. 1. The MiniPERM bioreactor.

with semi-permeable membranes and fermentation systems. Flasks tend to be cultured statically whereas fermentation requires mechanical agitation of the cells. The MiniPERM bioreactor is our method of choice for the high-density culture of hybridoma cells **(Fig. 1)**.

3.2.1. The MiniPERM Bioreactor

The modular system, consisting of a 40 ml disposable culture chamber and a 550 ml reusable nutrient module separated by a dialysis membrane (12.5 kD molecular weight cut-off), allows for the production of a low-volume, high-density cell population with a correspondingly high antibody yield.

The hybridomas and secreted antibody are retained in the production module **(Fig. 2)**. Depending on the cell line, cell densities can reach upward of 1×10^7 cells/ml. The MiniPERM bioreactor is rotated on a universal turning device that allows four MiniPERM bioreactors to be run simultaneously for larger production runs of one hybridoma or production of different hybridomas. This motion speeds the distribution of nutrients to the cells and facilitates the removal of metabolic waste products and CO_2-processes that rely on passive diffusion in static

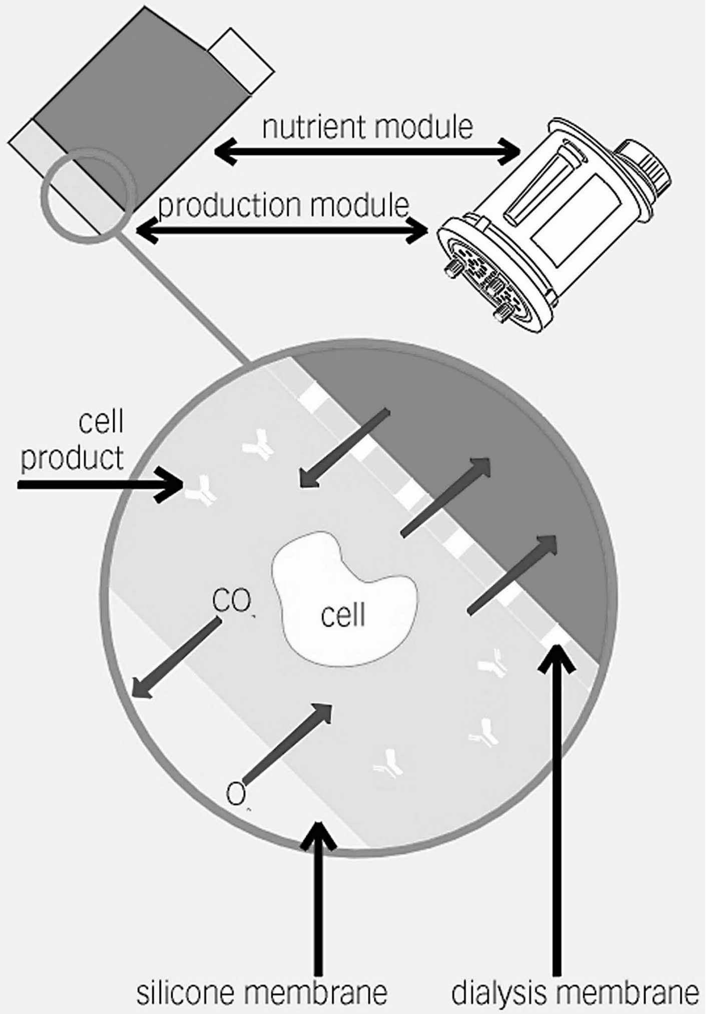
nutrient module
production module
cell
product
CO₂
cell
O₂
silicone membrane
dialysis membrane

Fig. 2. The MiniPERM production module.

bioreactors. Sample collection and harvesting is via Luer-Lock connections on the production module. The MiniPERM is a continuously fed culture system that allows cultures to be maintained for several weeks.

3.2.2. Inoculation of the Bioreactor

Assemble the MiniPERM bioreactor according to the manufacturer's instructions (*see* **Note 9**), disinfect the universal roller and install in a CO_2 incubator. Media for the production module (production medium) and the nutrient module (nutrient medium) should be prepared prior to inoculation (*see* **Notes 1–4**). The production medium is complete medium as described previously but supplemented with a further 5% FBS and 0.1% v/v CellPROTECT (*see* **Note 10**). The nutrient medium contains the same components as complete medium, with the exceptions of 5% FBS instead of 10% FBS and with the addition of 0.2% v/v AntiFOAMa (*see* **Note 11**). Both the nutrient media and the production media are prewarmed to 37°C to prevent expansion of the MiniPERM membrane when introduced into the incubator.

1. Detach the hybridoma cells by tapping and gentle pipetting from a 225 cm^2 tissue culture flask, wash by centrifugation at 300 × g for 10 min and resuspend the resulting cell pellet in 5 ml production medium.
2. Calculate the cell density by diluting 10 µl of suspension in 90 µl Nigrosin then count the cells using a haemocytometer. Adjust the density to 5×10^5 cells/ml in production medium and inoculate the reactor with 35 ml of this suspension using the following procedure (*see* **Note 12**).
3. Draw the suspension slowly up into a 50 ml hypodermic syringe (*see* **Note 13**). Unscrew the two Luer-Locks on the assembled MiniPERM and inject the cell suspension carefully into the production module while the module is slowly rotated (*see* **Note 14**). This ensures that displaced air is expelled through the open Luer-Lock.
4. Replace both Luer-Locks then add 400 ml of nutrient medium to the nutrient module. Close the cap and place the MiniPERM module inside an incubator on the Universal turning device set at 5 rpm (*see* **Note 15**).

3.2.3. Sampling and Harvesting

1. Remove 1 ml samples daily using a 2 ml syringe to assess the culture (*see* **Note 16**). To do this, put the MiniPERM on a stand and turn it so that a Luer-Lock sampling port is at 12 o'clock. Open the port and insert the 2 ml syringe (*see* **Note 17**). Rotate the device 120 degrees clockwise so that another port is at 12 o'clock and the sample port used for withdrawal is now at 4 o'clock.
2. Assess cell viability using Nigrosin exclusion (*see* **Note 18**).
3. Harvesting is performed using the method described for sampling, with the exception that a larger quantity of medium is removed using a 50 ml syringe. Harvest 20 ml either when the cell viability falls below 50% or when the cell density exceeds 2×10^7 cells/ml. The remaining 15 ml of cell suspension allows for repopulation of the production module following the addition of 20 ml fresh production medium.
4. Change the medium in the nutrient module every 2 to 4 days (*see* **Note 19**). Proceed with harvesting and replacement of nutrient media in a cyclic manner.
5. After sampling and harvesting the Luer-Locks must be wiped down with 70% ethanol to prevent infection being introduced to the production module (*see* **Note 20**). Periodical measurement of cell-density, viability, and antibody production over the course of the production run will help in the planning of future production runs.

3.3. Storage of Hybridomas

3.3.1. Cryopreservation

Prepare freezing mix (FM) in advance of the procedure. To do this, mix 10 ml FBS with 8 ml CM and 2 ml DMSO and store at 4°C (*see* **Note 21**).

1. Harvest the cells and supernatant from five 225 cm^2 flasks one day after being seeded (*see* **Note 22**) and pool the resulting cell suspension.
2. Remove 2 ml of this suspension and add to a 25 cm^2 flask. Supplement with 3 ml fresh CM and place the flask in the incubator (*see* **Note 23**).
3. Centrifuge the remaining suspension at 300 × *g* for 10 min at 4°C. Retain the supernatant for antibody analysis. From this stage onward all reagents should be used at 4°C. Recover the cell pellets, pool the cells, resuspend in 50 ml CM and wash by centrifugation at 300 × *g* as before.
4. Discard the wash supernatant and resuspend the cell pellet in 20 ml fresh CM. Calculate the cell density by diluting 10 µl of suspension in 90 µl Nigrosin and counting the cells using a haemocytometer.
5. Centrifuge the cells as before then finally resuspend the pellet to a density of 5×10^6 viable cells/ml in FM and 1 ml added to each cryovial.
6. Place the vials in a Nalgene freezing tub filled with 2-propanol then place in a −70°C freezer overnight (*see* **Note 24**).
7. Transfer the vials to LN_2 for long-term storage (*see* **Note 25**).

3.3.2. Resuscitation of Frozen Cells

1. Remove one vial from the cell bank in LN_2 after 24 h of storage and immediately thaw by immersion in a water bath at 37°C (*see* **Note 26**).
2. As the last of the ice disappears, transfer the suspension to a centrifuge tube (*see* **Note 27**). Slowly add 10 ml CM at 4°C to the cells with gentle mixing and centrifuge the suspension at 300 × *g* for 10 min at 4°C.
3. Wash the cells by resuspending the pellet in 10 ml CM at 4°C and then centrifuging as before.
4. Finally resuspend the cells in 1 ml CM and count as described in **Subheading 3.3.1**.
5. Adjust the cell concentration to 2×10^5/ml in CM, add to a 25 cm^2 flask and incubate as described in **Subheading 3.1.2** (*see* **Note 28**).
6. Visually monitor the cells daily for growth and lack of contamination. Expansion of the cells can be taken as evidence of a viable cell bank and also that no microbial contamination of the cells occurred during the freezing/resuscitation process (*see* **Note 29**).

3.3.3. Management of a Cell Bank

Validated cell banks are essential for the long-term survival of hybridomas. Ideally, databases should be set up to manage the banks and to record information about the cells. An example of useful fields for a database are set out in

Hybridoma identification code		
Specificity		
Isotype		
Cloning history		
Passage number		
Result of *Mycoplasma* screen		
Date frozen		
Cell density per vial		
Number of vials in bank		
	Date	Viability
Resuscitation (1 week)		
Resuscitation (1 month)		
Resuscitation (6 months)		
Resuscitation (1 year)		
Resuscitation (2 years)		
Resuscitation (3 year)		
Resuscitation (4 years)		
Resuscitation (5 years)		

Fig. 3. Recommended fields for management of hybridoma cell banks.

Fig. 3. One vial of cells should be resuscitated after one week, one month, six months and then annually and checked for viability following the procedure set out in **Subheading 3.3.2**. A new cell bank should be made if there is concern about the viability of the stock. It is recommended to have vials stored in more than one container to safeguard against loss as a result of mechanical failure of the storage system.

4. Notes

1. It is not desirable to routinely use antibiotics in tissue culture. Antibiotics may limit growth of contaminants without eradicating them, ultimately having a detrimental effect on the cells. Sound tissue culture technique

conducted in suitable facilities with appropriate equipment is sufficient to maintain established cell lines. However, hybridomas are expensive to produce and are often unique, so the inclusion of antibiotics in the early expansion stages acts as an added safeguard against contamination.

2. CM should be prepared at least three days before use to allow for sterility checking. CM can be stored for two weeks at 4°C, after which time the components will deteriorate. Glutamine is notably labile, hence storage of the concentrated stock at −20°C. It is possible to purchase a formulation of glutamine supplement that is stable (cat. no. 35050-038; Glutamax™, Gibco Invitrogen Corporation).
3. Selection of appropriate FBS is crucial for successful growth. Batches of FBS should be screened prior to selection for their ability to support hybridoma cloning and growth. Antibody content of FBS tends to be low, however batches of FBS may contain antibodies to ruminant pestiviruses and other transplacental pathogens. In the vast majority of cases this will not present a problem unless the hybridomas are producing MAbs to be used for pestivirus diagnosis or research. If the FBS contains pestivirus itself, this will create problems if the MAbs are used for research involving ruminant cells that can be infected by virus that then establishes a persistent infection and cannot be eradicated *(7)*.
4. Hybridomas vary in their growth characteristics and different lines will multiply at different rates. The expansion of hybridomas after they have been cloned requires careful monitoring to ensure that the cells have suitable growth conditions and an adequate supply of nutrients. Daily examination is desirable, with good note-keeping to record growth patterns. Cell populations are expanded by a gradual step-wise increase in the size of the culture vessel and volume of medium as the cell number increases. A change in the colour of the medium from orange to pale yellow signifies a drop in pH as the cells metabolize. This is accompanied by an increase in cell density and indicates that the cells can be moved to a larger culture vessel with fresh medium. If the medium becomes bright yellow the cells require urgent attention. CM should be warmed to 37°C before being added to cells. If cells are at low density and/or showing signs of poor growth, CM can be temporarily supplemented with OPI solution to a final concentration of 2% to encourage cell growth. Cell counts should be performed if required (*see* **Subheading 3.2.2**). Hybridoma growth can be modulated by altering the concentration of FBS in the CM, with lower concentrations reducing the rate of growth. This may be appropriate if daily monitoring is not feasible, but this may not be suitable for every hybridoma.
5. Hybridomas vary in their ability to adhere to plastic and to each other. It is important to avoid subjecting the cells to excessive mechanical stress such

as vigorous pipetting or foaming of the medium that can cause damage and cell death when removing them from plastic or disrupting clumps.

6. Culture flasks with vented filter caps allow for gaseous exchange between the culture and the incubator while protecting the cells from airborne microbial contamination.
7. There are various methods for detecting *Mycoplasma* contamination of cell culture. A sensitive PCR test with broad specificity for *Mycoplasma* species is available ***(8)***. Our method of choice is an enzyme immunoassay that detects four species of *Mycoplasma* (cat. no. 1296744; Roche Diagnostics Ltd, Charles Avenue, Burgess Hill, UK). There are several products available for the eradication of *Mycoplasma* species from cell lines. The effectiveness of the treatment will depend on the cells and involves trial-and-error. This is because some cell lines are very sensitive to the chemicals used to eradicate *Mycoplasma* and may become static or die during treatment. We use *Mycoplasma* removal agent (MRA) according to the manufacturer's instructions (BUF035; AbD Serotec, Langford Business Park, Kidlington, Oxford, UK).
8. Once hybridomas cell lines have been cloned they tend to have a stable phenotype. However, they do divide rapidly and can spontaneously change. Cells that do not produce antibody can arise from cloned populations, albeit at a low frequency. The growth properties of non-producing cells may not be the same as that of the parent clone, potentially outgrowing the producing cells which would resulting in loss of the hybridoma line. An important rule to remember is that different hybridoma lines should never be handled simultaneously in tissue culture. There is the risk of cross-contamination and if this does occur, it may not be immediately apparent if the lines are phenotypically similar (e.g., adherence properties etc). As with reverting clones, contamination may only become apparent when the supernatant is tested for MAb content. Hybridoma lines can be safeguarded by ensuring that cells are cryopreserved at an early stage after cloning (**Subheading 3.3**), by routine checking for MAb production, isotyping and sub-cloning if required. Mouse monoclonal antibody isotyping kits can be obtained from Roche (cat. no. 1 493 027; Roche Diagnostics Ltd).
9. Pre-assembled sterile bioreactors are available from the manufacturer. With the classic kit the production modules are sterile and can be used just once, the nutrient module needs to be assembled and sterilized by autoclaving.
10. Some hybridomas require a higher concentration of FBS, up to 15%. As the cells in the production module are subject to shearing forces due to the rotation of the bioreactor, CellPROTECT can be used as an antishearing supplement.
11. Nutrient medium is supplemented with 5% foetal bovine serum. Low molecular weight serum proteins diffuse across the dialysis membrane

between the nutrient and production modules. A reservoir of low molecular proteins is required in the nutrient module to maintain the equilibrium for hybridoma growth and survival. Accumulation of foam in the nutrient module can be a problem. To counteract foaming, do not exceed a concentration of 5% FBS in the nutrient module and add AntiFOAMa anti foaming agent. Do not fill the nutrient module with more than 400 ml of nutrient medium. An air space is required within the module to ensure successful hybridoma growth.

12. Hybridomas for inoculation into the MiniPERM bulk culture device should be greater than 90% viable and free from infection with *Mycoplasma* or other microbial agents. Ideally, a concentration of 5×10^5 cells/ml is required for the 35 ml inoculum, although the density can be increased to 2×10^6 cells/ml if the culture fails to seed at the lower density.
13. Do not use a needle for loading the syringe since the shearing forces can damage the cells.
14. Work on the MiniPERM should be carried out promptly in order that the cells do not settle and clump together impairing their viability. This is particularly important to bear in mind when high densities are reached as these populations will rapidly exhaust nutrients and oxygen when static. In addition the localized build up of metabolic products will have a toxic effect on the cells. A stand is available to aid this procedure.
15. Mouse hybridomas can be turned at speeds of 5–20 rpm. Cells sensitive to shearing forces should be turned at lower rpm.
16. To maintain the optimum cell density, cell viability and maximize the antibody yield within the cell chamber it is necessary to sample the cell population at regular intervals. The frequency of harvesting is dependent on the growth properties of the hybridoma being cultured so sampling should initially be performed daily to assess the culture.
17. Pressure within the module can cause the cell-culture medium to spurt out when the Luer-Locks are opened. If the rubber membrane of the production module is distended stand the MiniPERM upright and release the cap of the nutrient module and gently push the membrane into a flat position.
18. Cells often take a time to adjust to the bioreactor and a small initial drop in viability is normal. If the cells are not growing and the viability has dropped below 50%, and contamination has been ruled out, there are several options to try: (a) reseed the bioreactor at a higher cell density; (b) adjust the rolling speed; (c) increase the serum concentration in the nutrient module; (d) add OPI at 1/50 to promote cell growth.
19. Low-density cultures (e.g., 5×10^5 cells/ml) will require fresh nutrient media less frequently than high-density (e.g., 5×10^6 cells/ml) cultures. A change in the colour of the medium from orange to yellow indicates that the medium requires to be changed.

20. Replacement sterile caps for the nutrient module Luer-Locks can be used to reduce the chances of contamination.
21. The components of FM (FBS, CM, DMSO) are used in the ratio of 5:4:1. It is preferable to prepare fresh FM for each cell bank and chill to 4°C. DMSO can be purchased in 10 ml units. Once a vial has been opened, any unused DMSO can be stored at 4°C in a sealed container. Undiluted DMSO crystallizes at 4°C, so it is recommended to store aliquots in suitable working volumes to avoid repeat thawing of the stock.
22. Cells are in optimal condition for cryopreservation when they are in log-phase growth. This occurs when the cells are at low-to-medium density in CM that is not exhausted of nutrients.
23. It is advisable to maintain a small culture of cells until the viability of the cell bank is validated. This ensures survival of the line should the freezing procedure fail.
24. To avoid cell damage due to rapid formation of ice crystals a freezing rate of 1°C per min is optimal. If Nalgene tubs are not available, vials can be wrapped loosely in cotton wool to achieve a similar effect, but this has a more variable success rate.
25. Appropriate safety procedures must always be followed when dealing with LN_2 storage. Cells can be stored in liquid phase or vapour phase, or in ultra-cool freezers. Prevention of ice-crystal formation is essential for successful long-term storage and the vials should be protected from temperature fluctuations wherever possible. Temperature fluctuations can damage cells when frozen, even if they do not result in the contents of the vial thawing out.
26. Cells are vulnerable in the transition period from freezing to resuscitation. Rapid thawing in a water bath is advisable to limit cell damage. The vial should never be submerged to the level of the seal on the cap, otherwise contamination will occur.
27. It is important not to allow the cells to remain in the water bath since they need to be washed free of freezing mix as soon as possible after they have thawed.
28. It is desirable to have a cell bank containing a high percentage of viable cells upon thawing. If the viability is less than 70%, another cell bank can be produced from the cells that have been kept in culture (**Subheading 3.3.1,** *see* **Note 23**). Hybridomas with low viability can be resuscitated into 96-well culture plates (1×10^5 cells/well), allowed to settle for 24 h, then the CM changed to remove intracellular products of dead cells that can inhibit the growth of the surviving cells. Alternatively, freshly-isolated syngeneic peritoneal mouse macrophages can be used as feeder cells for cell banks that prove difficult to resuscitate. The macrophages should be seeded at a

density of 5 × 10^3/well in a 96-well culture plate 24 h before resuscitation of the hybridoma. The macrophages will ingest cellular debris and release growth factors for the hybridoma.

29. If microbial contamination is observed, another vial should be resuscitated from the bank to confirm whether or not the contamination is unique to one vial. If contamination is found in other vials the bank should be discarded.

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Mouse Hybridomas as an Entryway to Monoclonal Antibody Design and Production

Eugene Mechetner

1. Introduction

Over the last 30 years, significant progress was made in the development of monoclonal antibodies (mAbs) initiated by the pioneer studies of Caesar Milstein on the structure and genetics of immunoglobulins in the 1960s and 1970s. **Table 1** summarizes the most significant advancements in the hybridoma field since the first publication by Kohler and Milstein in 1976 ***(1)***. The variety of research and diagnostic mAbs was expanded from "classic" mouse-mouse monohybridomas through the use of rat-rat, hamster-hamster and increasingly popular rabbit-rabbit monohybridomas ***(2–4)***, as well as relatively simple to make and efficient mouse-rat and rat-mouse heterohybridomas ***(2)***. After many years of persistent development complicated by difficulties in identifying reliable fusion partner cell lines, chicken ***(5)*** and, especially, human ***(6)*** monohybridomas are also coming closer to fruition. In the therapeutic arena, human mAb versions originated in transgenic mice ***(7)*** and recombinant mAbs expressed in vitro using phage displays ***(8)***, ribosome displays ***(9)*** and baculovirus expression vectors ***(10)*** are now playing a major role in the discovery and clinical applications of new immunotherapeutic agents. A new and rapidly evolving approach with a very promising future in multiple biomedical ventures is mAb manufacturing in non-animal system, including yeast ***(11)*** and plant ***(12)*** cells. Finally, fully synthetic mAbs prepared using amino acid ***(13)*** and nonamino acid ***(14)*** diversity platforms have been added to the arsenal of research, diagnostic and therapeutic mAbs.

However, the major challenge facing a neophyte is not in the selection of a specific source of antibodies or an expression system, but rather in gaining an overall understanding of the design and in circumventing experimental pitfalls

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Table 1
Brief summary of the monoclonal antibody field

Type of mAb	Description	Source	Reference
Animal, cell fusion based monohybridomas	mAbs produced by hybrids of immune cells and fusion cell line partner from the same animal species	mouse rat rabbit hamster chicken human	(1) (2) (3) (4) (5) (6)
Animal, cell fusion based heterohybridomas	mouse or rat mAbs produced by hybrids of the two species	mouse/rat, rat/mouse	(2)
Animal recombinant, in vivo	human mAbs produced in mice using antibody gene loci inserted in mouse germ line	mouse/human	(7)
Recombinant mAbs and their fragments (minibodies)	mAbs expressed in living E.coli	phage display	(8)
	mAbs expressed in bacterial or animal cell lysates	ribosome displays	(9)
	mAbs expressed and produced in vitro in the baculovirus system	insect	(10)
Non-animal (fungibodies, plantibodies)	mAbs produced by yeast cells in vitro	yeast	(11)
	transgenic mAbs produced in algae or higher plants (e.g., tobacco)	plants	(12)
Synthetic (multibodies)	mAb multimers created through chemical processes from multivalent recombinant fragments	amino acid based	(13)
	aptamers engineered synthetically using RNA or DNA ligands	non-amino acid based	(14)

common for beginners in the mAb trade. In this context, the original approach reported in 1976 ***(1)*** and virtually unchanged since then, remains a mainstay for the field and serves as a prime example of how a successful mAb ought to be developed. Consequently, this chapter is centered on the mouse monohybridoma methodology and its major objectives and limitations.

2. Materials

2.1. Animals

1. Host animals: BALB/c mice, 20- to 30-weeks old
2. Immunization: Complete Freund's Adjuvant (Sigma) and Incomplete Freund's Adjuvant (Sigma, St. Louis, MO)
3. Production of ascites: Pristane (2,6,10,14-Tetramethylpentadecane; Sigma)

2.2. Hybridoma Selection and Expansion

1. Myeloma fusion partner: P3X63Ag8.653 mouse myeloma (ATTC accession number CRL-1580)
2. Medium for routine cell maintenance: Iscove's (IMDM) or Dulbecco's (DMEM) Modified Eagle Medium, supplemented with 15% fetal calf serum, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 µM beta-mercaproehtanole, non-essential amino acids, and antibiotics of choice (e.g., penicillin/streptomycin, 50 units/50µg) (all from Invitrogen, Carlsbad, CA)
3. Medium for P3X63Ag8.653 before cell fusion: normal maintenance medium plus 8-azaguanine (Sigma, St. Louis, MO) at 15 µg/ml
4. Medium for routine protein-free production: PFHM II (Invitrogen) or UltraDOMA-PF (Cambrex, East Rutherford, NJ)
5. Medium for difficult-to-grow hybridoma clones: HybriCare Cell Culture Medium (ATCC, Manassas, VA).
6. Medium for hybridoma selection: normal maintenance medium plus HAT or HT using 500× concentrates of HAT and HT (69-X and 71-X, respectively; ATCC)
7. Cell fusion: polyethylene glycol solution (PEG) 40%–50 % (w/v), mol wt 1,450 (P7181; Sigma) in a protein-free medium or phosphate buffer saline (PBS)
8. Cell count: 0.4% Trypan Blue solution (Sigma)
9. Cell cryopreservation: hybridoma grade DMSO (dimethyl sulfoxide; Sigma)
10. Plastic ware: sterile 96-well plates, tissue culture flasks, Petri dishes, conical tubes (15 and 50 ml)

2.3. Monoclonal Antibody Characterization and Production

1. mAb isotyping: antibody-capturing strips kit (ISO1, Sigma)
2. mAb quantification: Standard Vectastatin ABC kit (PK-4000, Vector Labs, Burlingame, CA)
3. For complement-fixation testing: Low-tox rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada)
4. mAb concentration: for IgG supernatants, AMICON Centriprep 100 concentration units (Millipore, Billerica, MA)
5. Affinity chromatography: application specific mAb purification kits (Pierce Biotechnology, Inc., Rockford, IL)

Table 2
Timetable and example of immunization schedule

Day	Procedure	Route
0	Primary immunization	i.p.
14	First boost	s.c.
28	Second boost	s.c.
36	Serum collection; titration	from retroorbital sinus
42	Third boost	s.c.
56	Final boost	i.v.
59	Serum collection; cell fusion	total bleeding; spleen removal

3. Methods

3.1. Immunization

1. Immunization protocols may vary depending on the experimental model and objectives. A generic exemplar immunization schedule is shown in **Table 2**. Initial immunization: i.p. administration of 0.5 ml of Complete Freund's Adjuvant mixed at 1:1 (v/v) with antigen solution (5–100 µg of the antigen or 10^6–10^7 cells) (*see* **Note 1**).
2. Booster injections: s.c. administration (to increase the immune response, at multiple sites) of 0.5–1 ml of Incomplete Freund's Adjuvant mixed with 1–50 µg of protein or 10^5–10^6 cells.
3. Blood samples for subsequent serum titration are collected from the retroorbital sinus.
4. The total number of boost injections before the fusion should be determined using serum titration results.
5. Final boost: slow (to avoid anaphylactic shock) i.v. administration of 50–100 µl of purified antigen dissolved in PBS, or i.p. injection of the antigen.

3.2. Cell Fusion and Screening

1. Prepare P3X63Ag8.653 mouse myeloma in advance through propagating cells in flasks or roller bottles.
2. Grow myeloma cells in the presence of 8-azaguanine for at least 3 weeks (*see* **Note 2**).
3. Maintain myeloma cells in 8-azaguanine-free medium for 7 days before fusion.
4. Refresh medium 24 h before fusion. Harvest and wash the cells immediately before fusion, wash twice in ice-cold serum-free medium and keep on ice until adding PEG. Keep P3X63Ag8.653 cells viable and completely free of protein in the medium.
5. Keep 1.5 ml of 50% PEG solution in serum-free DMEM at 37°C until fusion.
6. After sacrificing the animal, collect blood through total bleeding, prepare serum samples, and keep 100 µl aliquots at −20°C for further analysis.
7. Remove and transfer the spleen into a sterile Petri dish with 10 ml of cold DMEM. Wash the spleen in two additional Petri dishes containing 10 ml of cold DMEM.

8. Use crusher/homogenizer containing 5–7 ml of cold DMEM to squeeze splenocytes from the spleen. Avoid circular motions of the pestle to maintain cell viability.
9. Remove the splenocyte suspension into a conical tube with ice-cold DMEM, gently pipet on the tube wall to break down large cell clumps, and collect debris-free splenocytes.
10. Wash twice at 200 g for 10 min with ice-cold DMEM.
11. Resuspend prewashed P3X63Ag8.653 myeloma and splenocytes cell pellets in 10 ml of cold DMEM and count cells using a hemocytometer.
12. Although different ranges of splenocyte-to-myeloma cells ratios and cell numbers have been reported, the approximately 1:1–1:5 ratios and a total of 5×10^7–10^8 cells per fusion worked best in our hands.
13. Combine splenocytes and myeloma cells in a 50-ml conical tube, resuspend in prewarmed to 37°C protein-free DMEM and spin down in a bucket rotor centrifuge at 200 g for 10 min to obtain a loose cell pellet (*see* **Note 3**).
14. Quickly aspirate the supernatant and dissociate the cell pellet by gently flicking on the bottom of the tube. Slowly add 1 ml of prewarmed PEG solution to the cell pellet, while gently tapping on the bottom to mix the cells with PEG.
15. Slowly add 40–45 ml of prewarmed at 37°C serum-free DMEM while gently swirling the tube after every 5 ml of DMEM added.
16. Wash once at 200 g for 10 min, remove the supernatant, gently pipet in 10 ml of prewarmed at 37°C complete HAT medium, and transfer into a container with another 190 ml of warm complete HAT medium.
17. Transfer the cells into 10 96-well flat-bottom plates, 0.2 ml per well (*see* **Note 4**).
18. Incubate at 37°C in 5% CO_2; replace one half of the well volume with complete HAT medium every two or three days.
19. Start screening 12 days after fusion or when wells containing hybridoma clones are 50% confluent. The screening procedure should yield prompt and definitive results to avoid excessive growth of hybridoma clones in their initial wells.

3.3. Hybridoma Cloning, Expansion, and Storage

1. Use at least three consecutive rounds of cloning.
2. Clone all positive wells after fusion in the initial 96-well plates.
3. Utilize the largest single colonies in subsequent rounds of cloning. For each initial colony, at least 15–20 single colonies should be tested and at least 10 best single colonies should be re-cloned, each into one flat-bottom 96-well plate (*see* **Note 5**).
4. Use Trypan Blue to determine the concentration and total number of viable hybridoma cells.
5. After 8–12 days of growth, select the largest 15–20 colonies and repeat the re-cloning procedure.
6. Cryopreserve all best colonies before the next round of cloning.
7. Utilize complete HAT medium for the first round of cloning (i.e., cloning of initial hybridoma colonies).
8. In the second round of cloning, start replacing 50% of the well volume with complete HT medium every 2–3 days.

9. In the third round of recloning, start replacing 50% of the well volume with normal complete DMEM using the same timetable.
10. Repeat testing of the supernatants and cell cryopreservation at each recloning step (*see* **Note 6**).
11. To expand the best hybridomas, transfer cells into 24-well plates and, when stable growth is achieved, into T25 and T75 flasks. Test and cryopreserve clones from each selection/adaptation cell culture step.
12. After stable growth of hybridoma population is well established, proceed to mass production in multiwell plates, tissue culture flasks, roller bottles, cell factories, etc.
13. Hybridomas are cryopreserved using standard freezing procedure in 90% fetal bovine serum mixed with 10% DMSO, 10^6–10^7 viable cells per vial.
14. Thaw hybridoma cultures at 37°C, followed by a transfer of cells into conical tubes, drop-wise addition of complete hybridoma medium, gentle centrifugation at 200 g for 10 min, and addition of 2 ml of complete medium to cell pellets. Transfer resulting cell suspension into 24-well plates containing full hybridoma medium.

3.4. Isotype Determination

1. The isotype of a mAb is a major parameter to consider its performance in end-use applications. mAb candidates should be isotyped at early stages of the hybridoma selection/expansion process.
2. Various isotyping kits available commercially can be used for primary and advanced hybridoma screening. We recommend a quick (1 h) and sufficiently sensitive (1 µg/ml) Immunotype ISO1 Mouse Monoclonal Antibody Isotyping Kit (Sigma) based on the treatment of nitrocellulose strips with 2 ml of a hybridoma supernatant.
3. Although over 90% of murine immunoglobulins contain kappa light chains, the kappa vs lambda light chain composition of each candidate mAb should be also determined.

3.5. Complement Fixation Testing

1. Use Rabbit Low-Tox-M Complement to test the ability of mAb candidates to fix and activate complement.
2. mAb concentration in the tested material should be equal or greater than 1 µg/ml, as determined by ELISA based Standard Vectastatin ABC kit.
3. Adjust 1 ml of mAb solution in serum-free hybridoma medium to pH 7.2–7.4 and incubate with 10^6 washed target cells at 4°C for 1 h. Wash twice with serum-free medium, incubate pre-treated cells with 1 ml of pre-diluted rabbit complement for an additional 1 h at 37°C, and calculate the percentage of dead cells treated with the mAb vs negative control (hybridoma medium with no mAb). Repeat two to three times using different lots and concentrations of the complement.

3.6. Ascitic Fluid Production

1. Inoculate (i.p.) BALB/c mice with 0.5 ml of Pristane.
2. Seven days after Pristane injection, inject (i.p.) 5×10^6 to 5×10^7 hybridoma cells pre-washed in serum-free medium.
3. Subsequently, check inoculated mice for abdominal distension every 3 days until the abdomen is filled with ascitic fluid.
4. Drain ascitic fluid from the abdomen.
5. Centrifuge the fluid at 500 g for 10 min and remove the fat and clotted material (*see* **Note 7**).
6. Aliquot and keep at −20°C until further analysis.
7. One BALB/c mouse can produce up to 10 ml of ascitic fluid, with mAb concentration of 5–10 mg/ml (*see* **Note 8**).

3.7. Antibody Production in Protein-Free Medium

1. After the third round of cloning and subsequent clone expansion, gradually replace the original hybridoma medium with PFHM II supplemented with 15% fetal calf serum. Culture cells in 25 or 75 ml flasks.
2. Gradually reduce the concentration of fetal calf serum to 0% by replacing complete PFHM II with serum-free PFHM II.
3. Re-clone the hybridoma population by serial dilution in serum-free PFHM II in 96-well plates, re-test for mAb production, expand and cryopreserve the best clones.
4. To achieve high mAb titers (up to 0.5 mg/ml), passage in PFHM II medium in plastic flasks, cell factories or roller bottles (*see* **Note 9**).

3.8. Antibody Purification

1. Depending on the experimental system and intended end-use applications, commercially available purification kits, mostly protein A, G and L based, are used to purify mAbs from hybridoma supernatants and ascites. Proteins A and G absorb the Fc portion of mouse mAbs, at pH 8.2 (Protein A) and pH 5 (Protein G).
2. All mouse IgG subclasses, except IgG1, can be purified using either of these two systems. Because of better stability of mouse mAbs at slightly basic pH, Protein A based purification systems are more advantageous than those based on Protein G.
3. Mouse IgG1 and IgM mAbs can be isolated using Protein L based kits.
4. Refer to Pierce's Web site www.piercenet.com for many useful mAb purification protocols.

4. Notes

1. An efficient immunization protocol, which avoids the neutralization of the antigen by circulating antibodies and thereby resulting in high numbers of proliferating antibody-secreting B-cells is essential in generating high-quality mAbs. Immunization schedules have to be optimized for each

antigen and end-use application, based on intermediate titration data and cell fusion outcomes.

2. It is critical that P3X63Ag8.653 cells remain resistant to 8-azaguanine and not capable of producing HGPRT to re-utilize hypoxanthine contained in hybridoma selection media. To eliminate HGPRT revertants and facilitate after-fusion growth of hybridoma clones, culture P3X63Ag8.653 cells in a medium supplemented with 8-Azaguanine and (in some protocols) HT.
3. Avoid protein contamination of PEG stock solutions and thoroughly wash splenocytes and P3X63Ag8.653 cells in protein-free medium. Free protein adversely affects the cell fusion process.
4. Unused mouse splenocytes can be cryopreserved and utilized later in additional cell fusions to improve end-use parameters of the desired mAb. Furthermore, additional fusions can be used to create heterohybridomas with myeloma cell lines from different animal species.
5. Re-test hybridoma clones periodically to retain mAb production and prevent overgrowing by hybridoma revertants which produce no mAb. Continued (over two months) expansion of pre-selected hybridomas is not recommended. Instead, new rounds of mass hybridoma expansion should be initiated using confirmed frozen cells from earlier passages.
6. As is the case with immunization schedules, all mAb screening protocols and procedures have to be optimized in preliminary experiments. It is imperative that screening be performed to address the intended end-use application. For example, if you need a mAb for flow cytometry staining of cell surface epitopes, it would not make sense to use western blotting or ELISA to select hybridoma clones. Such seemingly straightforward strategies rarely work well in real life, resulting in developing hybridomas producing superfluous mAbs.
7. After separation from ascitic fluids, living ascitic cells can be cryopreserved and used to generate more ascites. However, because of potential overgrowth by non-producing clones, ascitic cell populations cannot be passaged more than twice.
8. Although still hugely popular, production of large amounts of mAbs from mouse ascitic fluids is not, in most cases, the best possible approach. Manufacturing mAbs from tissue culture supernatants, especially using serum- or protein-free media, is a better alternative resulting in large-scale mAb preparations free of non-specific mouse immunoglobulins.
9. In many situations, it is worth investing additional effort in selecting hybridomas which can grow as suspension cultures. This ability makes hybridoma cell lines particularly suitable for industrial-scale production and, if needed, for using tissue culture supernatants without additional concentration and purification.

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Potential Pitfalls in Monoclonal Antibody Development and Applications

Eugene Mechetner

1. Introduction

The field of monoclonal antibodies (mAbs) continues to expand upon its phenomenal success in producing valuable research tools and clinically potent immunological drugs with enhanced therapeutic window. In 2006, the worldwide revenues of clinical mAbs reached $20.6 billion. Currently, over 200 biotech companies are pursuing clinical mAb projects through R&D investment, corporate acquisitions, and licensing transactions ***(1)***. The same trend, albeit on a smaller scale, can be seen in the research and diagnostics arenas, where mAbs are widely used as irreplaceable tools for targeted detection, quantification, and imaging ***(2)***.

These significant achievements, combined with the relative ease of use of the currently well developed mAb technologies, attract a large number of new researchers from academia and industry. Increasing amounts of information are being generated, and the quality of research sometimes does not match the high standards of the field. This predicament is confounded by some bioreagent companies trying to cut corners to improve their profit margins in this very lucrative marketplace. Therefore, one of the major challenges for the newcomers is clear understanding of potential pitfalls and mistakes to avoid when developing and using mAbs tailored to specific end-use applications.

This chapter briefly describes the most common obstacles, and some solutions to them, in the design, development, and applications of mAbs. As shown in **Table 1**, these problems arise at each step of the mAb development process and should be understood and addressed promptly in order to maintain the project's momentum. Our goal is not to describe every possible experimental scenario, but to show the logic of the process and call for constant vigilance to successfully meet new and on-going challenges.

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Table 1
Potential Pitfalls and Their Origin in the mAb Development Process

Problem Area	Specific Problem	Problem Origin	Cross-References in the Text
primary mAb characteristics	undesired specificity	inadequate immunization and clone selection protocols, resulting in mAbs with superfluous specificities and broad cross-reactivity	3.1. Development of the UIC2 Hybridoma; 3.2. UIC2 mAb Selection; 3.3. UIC2 mAb Characterization (*see* **Notes 1– 9, 14**)
	low sensitivity	low affinity mAbs due to insufficient selection between mAbs with the desired end-use specificity	
	cross-reactivity	inadequate or insufficient characterization of the pre-selected mAbs, resulting in mAbs directed against epitopes shared with other targets	
	target epitope limitations	suboptimal binding to epitopes of the antigen of choice due to flawed design of mAb selection and characterization protocols (e.g., ELISA vs IHC)	
mAb production and handling	cross-reactivity when using mAbs purified animal sources	in vivo contamination by animal immunoglobulins (e.g., from ascites or serum) and other reactive proteins	3.4. UIC2 Production and Handling (*see* **Notes 10, 11**)
	cross-reactivity when using mAbs derived from tissue culture supernatants	in vivo contamination by immunoglobulins from tissue culture media supplemented with animal sera	
	lack of reactivity in mAb preparations	loss of mAb secretion or overgrowth by non-producers due to insufficient re-cloning or passaging hybridoma cultures for extended periods of time	
	cross-reactivity with unrelated antigens	cross-contamination by unrelated mAb producing cells	
	low sensitivity after mAb purification	reduced mAb reactivity resulting from inadequate purification protocols (e.g., very low pH)	
	decrease or loss of activity during storage and transportation	loss of mAb reactivity due to flawed storage or transportation procedures	

mAb logistics	desired mAb is unavailable	restricted access to intellectual property or proprietary ownership limitations	3.5. UIC2 Commercialization and Logistics (*see* **Notes 12–14, 16**)
	poor performance by mAbs provided by commercial suppliers	multiple reasons, stemming mostly from low internal validation and insufficient QA/QC standards	
	mAb performance inconsistent with its specifications or associate protocols	incorrect information provided by the mAb source or related scientific literature	
	prohibitedly high cost of the desired mAb	mAb price limitations due to excessive monopoly in the marketplace, patent fees, high production expenses, or other commercial considerations	
mAb end-use applications	inconsistent or poor performance of the mAb of choice	wrong choice of the mAb, or flawed mAb information, or insufficient internal validation	3.6. UIC2 end-use applications: the UIC2 Shift Assay (*see* **Notes 8, 14–16**)
	poor performance of the chosen protocol	suboptimal choice of experimental modalities, or flawed mAb protocol information	
	poor performance of the chosen detection system	incorrect choice of the detection system or antigen treatment/retrieval methodology	

Throughout the chapter, we discuss our studies on human multidrug resistance protein pump, P-glycoprotein (Pgp), encoded by the MDR1 gene. Since only 7% of the protein is exposed on the membrane, it had been proven notoriously difficult to generate mAbs against external epitopes of human Pgp ***(3)***. Despite multiple efforts, only a handful of mAbs reactive with extracellular epitopes had been developed, and none of these mAbs exhibited functional activity. In 1992, we described a mouse mAb, UIC2, that recognized extracellular conformational epitopes of human Pgp, inhibited the efflux of compounds transported by Pgp from MDR1 cells, and reversed the in vitro resistance to MDR1 chemotherapy drugs ***(4–7)***. These studies are discussed in the context of the mAb development process.

2. Materials

Standard reagents, cell culture maintenance and fusion media, as well as buffers used for the development and characterization of the UIC2 mAb, are described in detail in this book in the chapter "Mouse Hybridomas as an Entryway to Monoclonal Antibody Design and Production."

2.1. Reagents

1. 25 μM vinblastine (Sigma, St. Louis, MO) solution was prepared in tissue-culture grade water, filtered through a 0.22 μ cellulose acetate unit (Corning, NY), and stored at 20°C.
2. Control chemotherapeutic compounds for vinblastine in the UIC2 shift assay: depending on solubility, stock solutions were prepared in water, DMSO, or RPMI-1640.
3. Control diluents for the UIC2 shift assay were added at the same final concentrations.
4. Fluorescent probes: 3,3-diethyloxacarbocyanine iodide, $DiOC_2$ and rhodamine 123 were purchased from Molecular Probes (Eugene, OR); Propidium Iodide (PI; Molecular Probes, Eugene, OR); all probes were diluted in 96% ethanol and stored at −70°C until use.
5. Radioimmunoprecipitation reagents: deoxycholic acid and phenylmethylsulfonyl fluoride (both from Sigma); ^{35}S-methionine (ICN, Costa Mesa, CA); immobilized protein A beads (Repligen, Cambridge, MA).
6. Sepharose-Protein A (Bio-Rad, Richmond, CA) was used for affinity chromatography.

2.2. Buffers

1. UIC2 shift Assay Buffer (AB): PBS supplemented with 2% fetal calf serum, FCS);
2. UIC2 shift assay Stop Buffer (SSB): PCS supplemented with 2% FCS and 0.01% sodium azide.

2.3. Cell Lines and Primary Cells

1. Mouse BALB/c 3T3-1000 cell line expressing human Pgp was derived by transfecting BALB/c 3T3 fibroblasts with human MDR1 cDNA in a mammalian expression vector pUCFVXMDR1, followed by a multistep selection of transfected cells for resistance to 1 µg/ml vinblastine ***(4)***.
2. Human K562/i cell line expressing human Pgp was developed using infection of human K562 leukemia cells with a recombinant retrovirus pLMDR1L6 carrying human MDR1 cDNA followed by subcloning, without cytotoxic selection, of high Pgp expressors, as determined by flow cytometry using functional MDR1 efflux assays ***(8)***.
3. Calibrating cell lines expressing pre-determined levels of Pgp (KB-8, KB-8-5, KB-8-5-11, and KB-V1) were selected from parental human KB-3-1 carcinoma by multistep selection with colchicine or vinblastine ***(1)*** (*see* **Note 1**).
4. Other cell lines utilized for the characterization of the UIC2 mAb were described in detail in (***refs. 4–8***).
5. UIC2 and UIC2/A hybridoma cell lines secreting the UIC2 mAb in complete or protein-free medium, respectively, can be purchased from ATCC (Accession No. HB11207 and HB11287) (*see* **Note 2**).
6. Peripheral blood lymphocytes (PBL) were isolated from buffy coats obtained from healthy volunteers by density gradient separation using Histopaque-1083 (Sigma, St. Louis, MO). PBL's were washed twice in RPMI-1640 and adjusted to10^6 cells/ml in RPMI-1640 for functional assays and flow cytometry analysis.

2.4. Antibodies

1. The following anti-Pgp mAbs were utilized to characterize the UIC2 mAb: MRK16 (IgG2a; kindly provided Dr. T. Tsuruo, University of Tokyo) directed against a human Pgp conformational epitope; HYB-241 and HYB-612 (both IgGl; a gift from L. Rittmann-Grauer, Hybritech), and mAb C219 (IgG2a; purchased from Centocor, Malvern, PA).
2. Affinity purified preparations of mouse myeloma protein UPC10 (IgG2a) were obtained from Sigma. All mAb samples were at least 95% pure IgG, as determined by SDS/PAGE. When necessary, mAbs were extensively dialyzed against phosphate-buffer saline (PBS) or Dulbecco's modified Eagle's medium (DMEM).
3. Unlabeled azide-free UIC2 mAb (mouse IgG2a) for Pgp functional assays can be purchased from Chemicon (MAB4334Z; Chemicon International, Temecula, CA) or purified using Protein A affinity chromatography from protein-free hybridoma supernatants (4). Phycoerythrin (PE) conjugated UIC2 can be also obtained from Chemicon (MAB4334PE) (*see* **Note 3**).
4. Control mAbs for flow cytometry were as follows: IgG2a-PE (negative mAb control to UIC2-PE)-from Sigma; IgG1-FITC and –PE, CD3-APC, CD4-FITC, CD8-FITC, CD14-FITC, CD15-FITC, CD19-FITC, CD45-FITC, CD14-PE, and CD54-PE-from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA); IgG1-APC, IgG2a-APC and CD56-FITC-from ExAlpha (Boston, MA); unconjugated purified IgG1, IgG2a, and IgG2b (negative controls to primary mAbs

used at matching concentrations in indirect immunostaining); PE-conjugated goat anti-mouse IgG2a (for secondary detection) from Caltag (Burlingame, CA).

3. Methods

The UIC2 mouse-mouse hybridoma was developed using standard protocols described in detail in this book in the chapter "Mouse Hybridomas as an Entryway to Monoclonal Antibody Design and Production," with some modifications introduced to address the specifics of the experimental system and match the properties of a desired anti-Pgp mAb to its intended end-use applications.

3.1. Development of the UIC2 Hybridoma

1. In order to optimize immunization and mAb selection protocols, we developed a mouse syngeneic cell line expressing high levels of human Pgp. Mouse BALB/c 3T3 fibroblasts (which were derived from and immunologically compatible with BALB/C mice used for hybridoma production) were transfected with human MDR1 cDNA using a mammalian expression vector (the pUCFVXMDR1 plasmid). The transfected cells were then additionally selected in growth media containing increased concentrations of vinblastine (up to 1 mg/ml). The resulting mouse cell line, BALB/c 3T3-1000, expressed high levels of human Pgp, and served as a well-matched (i.e. immunologically compatible with BALB/c mice, with the exception of human Pgp and. possibly, some other MDR related proteins) (*see* **Note 4**).
2. Syngeneic BALB/c mice were injected subcutaneously and intraperitoneally (i.p.) six times at two-week intervals with 10^7– 2 × 10^7 living and carefully washed BALB/C 3T3-1000 cells. The final boosting was done with 2 × 10^7 cells i.p., followed by slow injection of 5 × 10^6 10 cells intravenously to avoid anaphylactic shock. Four days after the last administration of BALB/c 3T3-1000, the spleen from one animal was removed and resulting splenocytes were fused with P3-X63-Ag8.653 partner myeloma cells.

3.2. UIC2 mAb Selection

1. Because our goal was to develop a functional mAb recognizing functional Pgp expressed on the outer cell membrane, we chose indirect immunofluorescence microscopy as the ultimate selection methodology for primary clones. Although more technically challenging than other high throughput techniques (e.g., ELISA), this methodology gave a direct answer to the experimental question at hand. Hybridoma supernatants were screened by indirect immunofluorescence microscopy, in parallel, on 12-well multitest slides (ICN) with living BALB/c 3T3 or BALB/c 3T3-1000 cells. One hybridoma clone of 556 clones tested produced a mAb, termed UIC2, which was reactive with Pgp expressing BALB/c 3T3-1000 but not with the parental BALB/c 3T3 cells (*see* **Note 5**).
2. A stable hybridoma line secreting the UIC2 mAb (IgG2aκ) was established through three additional rounds of subcloning by end-point dilution and screening of the

supernatant fluids. At each point in time, the fastest growing clones that produced the highest levels of UIC were selected in indirect immunofluorescence.

3.3. UIC2 mAb Characterization

1. UIC2 was purified from tissue culture supernatants and ascitic fluid by Sepharose-Protein A affinity chromatography. UIC2 mAb, tested by SDS-PAGE, was at least 95% pure IgG and did not fix rabbit complement in complement-mediated cytotoxicity tests.
2. To avoid nonspecific cross-reactivity from irrelevant mouse immunoglobulins, UIC2 purified from protein-free tissue culture supernatants was used for further mAb characterization (*see* **Note 6**).
3. As shown in **Table 2**, the UIC2 mAb reacted exclusively with human Pgp expressed on human and mouse cells and did not recognize mouse Pgp. UIC2 reactivity with Pgp from primate cells (including African green monkey) was later confirmed by immunofluorescence and IHC. Characteristically, UIC2 bound to human Pgp expressors in a dose-dependent fashion.
4. After UIC2 was characterized in morphological tests, a definitive proof of UIC2 specificity for human Pgp was provided by radioimmunoprecipitation (**Fig. 1A**). 5–10 × 10^6 BALB/c 3T3-1000 cells were metabolically labeled with 50 µCi/ml

Table 2
Characterization of the UIC2 mAb in Indirect Immunofluorescence.

Cell line	UIC2 reactivity
Human, selected for MDR	
KB-3-1 (parent)	–
KB-8	±
KB-8-5	+
KB-8-5-11	++
KB-V1	+++
CEM (parent)	–
CEM-VLB-100	++
Transfected with human MDR1 cDNA	
K562 (parent)	–
K562/Inf	+
BALB/c 3T3 (parent)	–
BALB/c 3T3-1000	+++
LR73 (parent)	–
LRMN1	+
Green monkey kidney (CV1-COS)	+
Mouse, selected for MDR	
J774.2 (parent)	–
J7-V2-1	–
J7-V3-1	–
J7-C1-100	–

From ***ref. 4***.

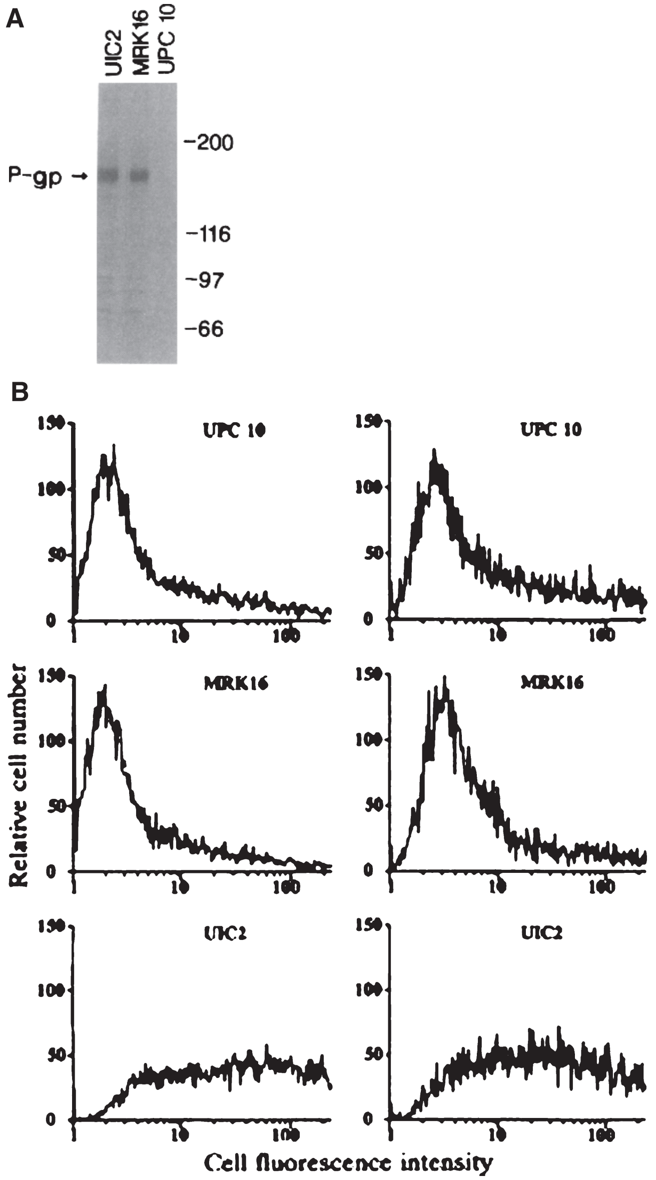


Fig. 1. Characterization of the UIC2 mAb in radioimmunoprecipitation (**A**) and flow cytometry (**B**) (from reference 4).

of ^{35}S-methionine in methionine-free DMEM supplemented with 10% FCS for 10–18 h. The cells were rinsed with PBS, and purified UIC2 was added in 2.6 ml of PBS to a final concentration of 20 µg/ml. After a one-hour incubation at room temperature, the cells were rinsed with PBS and then lysed with a detergent solution containing 0.2% deoxycholic acid and 0.2 µM phenylmethylsulfonyl fluoride in PBS for 2–4 min. The lysates were pre-cleared by microcentrifugation for 15 min at 4°C and incubated with immobilized protein A beads for one hour at 4°C. After five washes with the lysis solution, the precipitated protein was analyzed by SDS/PAGE in 7.5% gels.

5. The UIC2 mAb recognized a protein of 170–180 kDa expressed in MDR1 cells. This protein co-migrated in the gel with a band immunoprecipitated by another Pgp-specific mAb (MRK16) under the same conditions, thereby directly confirming that the antigen recognized by UIC2 mAb was human Pgp ***(4)***.
6. Solubilization with deoxycholate was critical for UIC2 immunoprecipitation. Solubilization with a more common detergent, CHAPS, allowed for efficient immunosedimentation of Pgp by MRK16 but effectively abolished the reactivity of Pgp with the UIC2 mAb. This result suggested (and later proved directly by epitope mapping) that UIC2 mAb and MRK16 mAb recognized different Pgp epitopes exhibiting different sensitivity to detergents (*see* **Note 7**).
7. Similarly to the MRK16 mAb, UIC2 did not react with denatured Pgp on Western blots, including conditions where human Pgp was detectable by the C219 mAb which recognizes an intracellular epitope of this protein ***(9)***. The choice of UIC2 characterization technique and the revealed target epitope restrictions were vital for decisive characterization of this mAb and its end-use applications (*see* **Note 8**).
8. The only remaining piece of evidence proving the functional nature of the UIC2 mAb was obtained in flow cytometry experiments on living multidrug resistant cells (**Fig. 1B**). 10^6 cells (K562/i or CEM-VLB100) expressing human Pgp were incubated with different anti-Pgp mAbs at 20 µg/ml in 3–5 ml of serum-free medium for 30 min at 0°C, washed twice, and loaded while with 0.5–1.0 µg/ml of Rh123 for 10 min for one hour. Rhodamine 123 (Rh123) is a mitochondrial fluorescent dye that is extruded from living cells by the Pgp membrane pump. 20 µg/ml of UIC2 was also added to the dye-free medium during the efflux period at 37°C. Dye retention after efflux was measured by fluorescence flow cytometry vs MRK16, or control IgG2a (mAb UPC10), or UIC2 pre-absorbed with anti-mouse IgG or anti-mouse IgM (negative isotype control) adsorbents.
9. UIC2 mAb did not alter the accumulation of a Rhodamine 123 at 0°C. However, UIC2 significantly inhibited subsequent efflux of Rh123 at 37°C. In sharp contrast, under the same conditions, MRK16 and two other anti-Pgp mAbs did not inhibit the efflux of Rhodamine 123 although the intensity of immunofluorescent staining of all tested cell lines by these mAbs was essentially the same. The inhibitory effect of UIC2 were abrogated by pre-absorption with the anti-mouse IgG (but not with control anti-mouse IgM) sorbent. These studies ***(4,7)*** directly demonstrated that the material responsible for inhibition of Rhodamine 123 efflux was the UIC2 mAb (*see* **Note 9**).

3.4. UIC2 Production and Handling

1. For clinical and commercial applications, we developed a UIC2 subclone, UIC2/A, capable of secreting high mAb concentrations in serum-free, protein-free culture media.

2. UIC2/A (ATCC accessioning number HB11287), was selected by gradually replacing the original growth medium with PFHM II Protein-Free Hybridoma Medium after the fourth round of recloning.
3. After one month of cultivation in the protein-free medium, UIC2 hybridoma cells had lost their ability to grow while attached to the flask surface. At that point, hybridoma cells were cloned by serial dilution in unmodified PFHM II in 96-well plates and supernatants from the best wells were tested for antibody production by indirect immunofluorescence. A clone that gave the strongest immunofluorescence signal was selected, transferred to a 25-ml flask, and the cell line was passaged as a suspension culture in PFHM II medium in 25-ml, to 75-ml, and finally to 175-ml flasks. When supplemented with 25 mM HEPES, UIC2/A could be cultivated in roller bottles, producing large volumes of medium with high UIC2 titers (0.2–0.4 mg/ml).
4. Indirect immunofluorescence was used to verify that UIC2/A hybridoma was secreting the mAb with the specificity and isotype (IgG2a) identical to those produced by parental UIC2 (*see* **Note 10**).
5. As shown in **Fig. 2**, the UIC2 mAb from UIC2/A supernatants was >80% pure without any purification (lanes 6–9). After a single-step purification on a Protein-A affinity column, the purity of the UIC2/A mAb was close to 100% (lanes 3 and 4), similar to that of the Protein A-purified mAb from ascitic fluids produced by parental UIC2 hybridoma (lanes 1 and 2).
6. UIC2 was stored in aliquots of 1–5 mg per vial at −20°C. Conveniently, this mAb is stable for months when stored under sterile conditions at 4°C, in PBS or culture medium at neutral pH at 0.5 mg/ml–10 mg/ml concentrations. In this form, UIC2 can be used for functional in vitro experiments.
7. The UIC2 mAb can be safely shipped in plastic vials at ambient temperature using overnight postal delivery or on dry ice for longer periods of time (*see* **Note 11**).

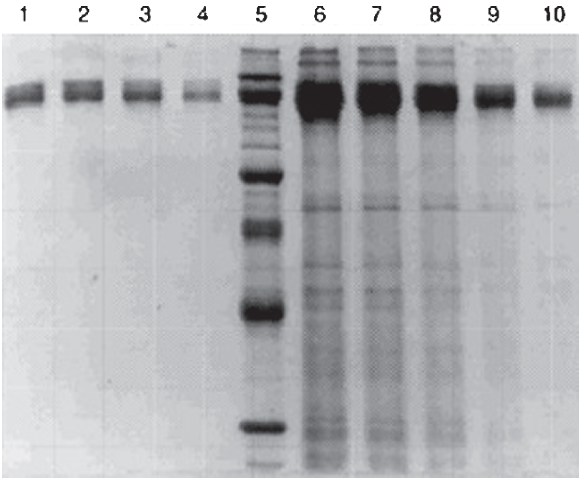
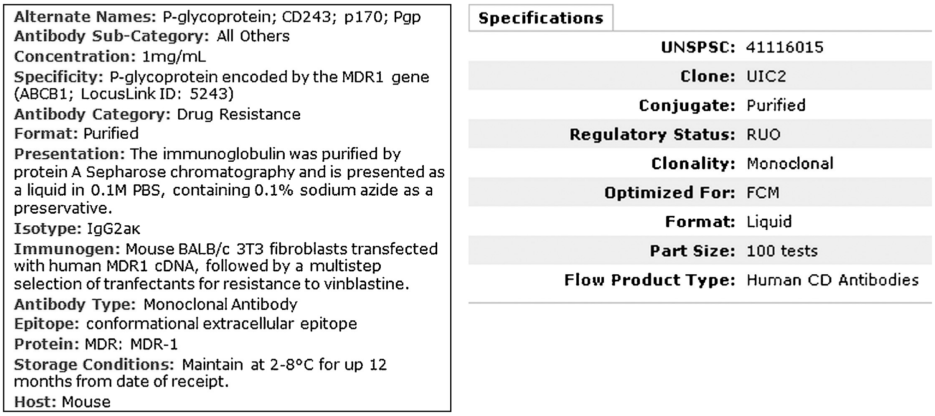


Fig. 2. Electrophoresis separation, under non-reducing conditions, of UIC2 and UIC2/A mAb preparations.

3.5. UIC2 Commercialization and Logistics

1. The ability of the UIC2/A cell line to produce high titers of the UIC2 mAb in protein-free media makes this cell line particularly useful for industrial-scale production.
2. When manufactured as a commercial research reagent by Chemicon International, gram amounts of the UIC2 antibody were readily produced from UIC2/A using the INTEGRA (Chur, Switzerland) cell line perfusion system and the Cellex (Minneapolis, MN) ACUSYST-miniMAX bioreactor (*see* **Note 12**).
3. In many applications (e.g., immunofluorescence and IHC staining, cell separation, immunoprecipitation) UIC2/A supernatants can be used without additional purification or concentration, resulting in major cost reductions without compromising the quality of mAb preparations.
4. The UIC2A mAb is currently available as a research reagent from Chemicon International (a division of Millipore Corporation), Beckman Coulter Inc., and Meridian Life Sciences, Inc., in the form of unconjugated and Phycoerythrin-conjugated preparations. Additionally, sterile and preservative-free UIC2 formulation and biotin labeled UIC2 are available from Chemicon (*see* **Note 13**).
5. One of the most convenient search engines to identify mAbs and find associated information and protocols is hosted by Biocompare, a free Web site at http://www.biocompare.com/jump/2045/Antibodies.html. A keyword search for "UIC2" would yield all commercial sources of the mAb and provide links to UIC2 product information (including pricing) and company product pages.
6. **Fig. 3** presents two examples of UIC2 product information and specifications from Chemicon and Beckman Coulter. Note detailed description of mAb presentation, concentration, immunogen, and storage conditions in Chemicon's specifications.



Alternate Names: P-glycoprotein; CD243; p170; Pgp
Antibody Sub-Category: All Others
Concentration: 1mg/mL
Specificity: P-glycoprotein encoded by the MDR1 gene (ABCB1; LocusLink ID: 5243)
Antibody Category: Drug Resistance
Format: Purified
Presentation: The immunoglobulin was purified by protein A Sepharose chromatography and is presented as a liquid in 0.1M PBS, containing 0.1% sodium azide as a preservative.
Isotype: IgG2aκ
Immunogen: Mouse BALB/c 3T3 fibroblasts transfected with human MDR1 cDNA, followed by a multistep selection of tranfectants for resistance to vinblastine.
Antibody Type: Monoclonal Antibody
Epitope: conformational extracellular epitope
Protein: MDR: MDR-1
Storage Conditions: Maintain at 2-8°C for up 12 months from date of receipt.
Host: Mouse

Specifications

UNSPSC:	41116015
Clone:	UIC2
Conjugate:	Purified
Regulatory Status:	RUO
Clonality:	Monoclonal
Optimized For:	FCM
Format:	Liquid
Part Size:	100 tests
Flow Product Type:	Human CD Antibodies

Fig. 3. Specifications for unconjugated UIC2 mAb preparations provided by Chemicon International (left panel) and Beckman Coulter, Inc. (right panel).

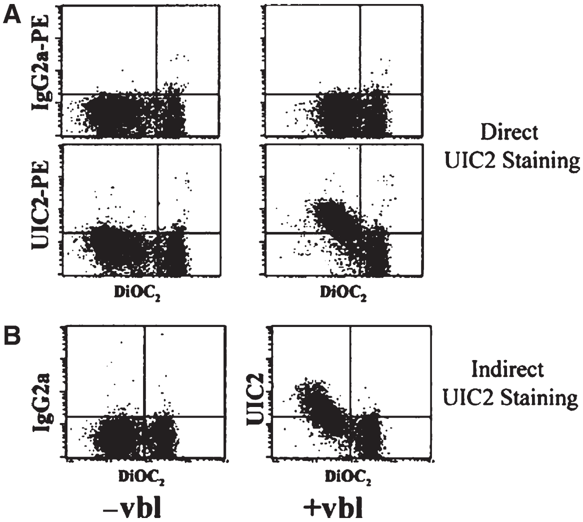
In contrast, Beckman Coulter's specifications lack most of the mAb specific description, including such essential information as UIC2 presentation and concentration. This information, or lack thereof, is critical for a researcher to identify the best source of the desired mAb and the most appropriate experimental and detection systems related to this mAb (*see* **Note 14**).

7. Similar Internet based information based approaches can be used to reduce costs associated with the mAb of choice. For example, list prices for 100 tests of unconjugated UIC2 are \$410, \$430 and \$597 from Chemicon, Beckman Coulter, and Meridian Life Sciences, respectively. Combined with other factors (such as product specifications, company's reputation, time of delivery, history of backorders, shipping costs, etc.), this information can be utilized by mAb users to optimize the quality and costs of their studies.

3.6. UIC2 End-Use Applications: The UIC2 Shift Assay

1. The UIC2 mAb is currently widely used by academic and industry researchers to detect human Pgp using flow cytometry, IHC, immunoimaging, and a variety of mAb based biochemical and cell based assays. Another important area of UIC2 uses is functional studies of Pgp in normal and transformed human cells.
2. Because UIC2 does not recognize mouse and rat Pgp's, this mAb can be successfully used to detect human Pgp in human xenotransplants without worrying about non-specific cross-reactivity from rodent tissue background (*see* **Note 15**).
3. Yet another advantage of using UIC2 in functional studies is its ability to enhance specific Pgp staining in the presence of Pgp-transported drugs at physiologic conditions. We found that the UIC2 mAb inhibited efflux of Rhodamine 123, $DiOC_2$, and many other fluorescent dyes and drugs transported by Pgp from MDR1 cells. Additionally, UIC2 reversed the in vitro resistance of MDR1 cells to Pgp-transported drugs in a dose-dependent fashion. Schinkel et al. demonstrated that observed variations in binding specificity between UIC2 vs other anti-Pgp mAbs correlated with functional differences in their ability to inhibit Pgp-mediated drug efflux ***(4–8)***.
4. Based on these phenomenona, we developed and validated a new flow cytometry test, the UIC2 Shift assay, which provides enhanced quantitative detection of Pgp expression and function in human MDR1 cells. The UIC2 shift assay will be described below as an example of valuable end-user application based on unique properties of the UIC2 mAb and potential pitfalls associated with this test.
5. UIC2 shift assay: load target cells (in this case, human PBL) with $DiOC_2$ at a final concentration of 1 μg/ml in RPMI-1640 on ice for 15 min in the dark. Immediately after loading, wash the cells twice in ice cold SSB, resuspend at 37°C in SSB, transfer to tissue culture flasks (10^6 cells in 10 ml), and allowed to efflux at 37°C in 5% CO_2 for 3–5 hr (typically, 15 min to one hour for high Pgp expressors and two to five hours for low Pgp expressors). Incubations at 4°C or with known Pgp substrates (e.g., vinblastine or verapamil) are to be included in the test as efflux background controls. After efflux, wash the cells twice with ice-cold SSB, and analyze by flow cytometry at 4°C in SB in the presence of 1 μg/ml PI.

Fig. 4. Direct (**A**) and indirect (**B**) flow cytometry analysis of human PBL with the UIC2 mAb (from reference 7).



6. As demonstrated in **Fig. 4**, UIC2 shift (i.e., enhanced Pgp staining in the presence of vinblastine) was observed mostly in DiOC$_2$-dim PBL in the direct and indirect versions of the assay. Pgp staining intensity was directly proportional to the degree of DiOC$_2$ efflux. No enhancement was observed in PBLs incubated with control IgG2a-PE in the presence of vinblastine at 37°C.
7. The intensity of indirect conventional UIC2 immunostaining (**Fig. 4B**, right graph) was, as expected, significantly higher than that of direct immunostaining with UIC2-PE (**Fig. 4A**, lower left graph). However, the UIC2 shift assay with directly labeled UIC2-PE only (**Fig. 4A**, lower right graph) produced Pgp immunostaining which was as sensitive as conventional indirect immunostaining. The latter observation exemplifies the ultimate end-use application of UIC2 to enhance the detection of human Pgp in living cells.
8. At all stages of the UIC2 shift development process and during its use in clinical diagnostics, all experimental protocols and reagents were internally validated and monitored for consistent performance (*see* **Note 16**).

4. Notes

1. The use of calibrating cell lines, i.e. a series of cell lines derived from one parental antigen-negative cell line and expressing different levels of the target antigen, is highly recommended at all stages of mAb development and characterization. Some examples of utilizing Pgp calibrating cell lines to validate UIC2 were presented in references 4 and 6.
2. ATCC (www.atcc.org) and other noncommercial institutions, such as the Developmental Studies Hybridoma Bank at the University of Iowa (DHSB,

http://dshb.biology.uiowa.edu/), can be extremely useful in all aspects of mAb research and applications. Not only these sources are publicly accessible internationally, but they also provide a wide variety of hybridoma cell lines at low prices that fit even the toughest research budget. In addition, consider requesting hybridoma cells or antibody preparations directly from the research group who developed and reported the mAb.

3. Azide-free mAbs, and preservative-free mAb preparations in general, are used mostly for functional studies and imaging in various in vitro models. These preparations are usually dialyzed against cell growth media, sterilized via filtration, kept sterile at 4°C for weeks and even months, and can be added to cell cultures directly from the mAb storage vial. Total protein concentrations should be maintained in the 0.5–10 mg/ml range using concentrated mAbs or inert carrier proteins (e.g., culture-grade bovine serum albumin).
4. Syngeneic mouse cells of BALB/c origin expressing the antigen(s) of choice are highly recommended for immunization and primary clone selection to generate mAbs against non-mouse functional epitopes. Some significant advantages of this approach include relatively safe and efficient immunization schemes, low numbers of irrelevant primary clones, and further uses of these cells for characterization and internal validation purposes.
5. Accurate choice of primary clone selection techniques is one of the most critical components of mAb development process. Some researchers and mAb companies attempt to cut corners and use deceptively simple and inexpensive clone selection techniques (i.e., ELISA when developing mAbs for IHC or flow cytometry protocols) that are incompatible with the end-use application. Such efforts usually result in the production of inadequate mAbs and, in general, waste of precious time and resources. The general rule of thumb is that your procedure for primary clone selection should be identical or very similar to your end-use application. In other words, if your objective is to generate a mAb that will work well in flow cytometry on live cells, choose the resolving flow cytometry or immunofluorescent techniques and avoid more simple, but not resolving ELISA.
6. Polyclonal immunoglobulins from blood and interstitial fluids, which are present throughout the body including ascitic fluids, as well as non-immune carrier proteins that bind non-specifically and unpredictably to a variety of antigens, are arguably the most common origin of cross-reactivity in research and, especially, commercial mAbs purified from mouse, rat, hamster and rabbit ascitic fluids. Another potential source of cross-reactivity is serum immunoglobulins from tissue culture supernatants when using adult bovine serum as a supplement. These contaminants essentially defeat the purpose of mAbs and should be avoided at all costs. Therefore, mAb

selection and production in serum-free, protein-free media are strongly recommended.

7. It is not unusual for mAbs directed against complex conformational epitopes to be completely inactive in Western blotting and other biochemical techniques resulting in "unphysiological" modifications of the target antigen. Choosing the right mAb characterization methodology (e.g., radioimmunoprecipitation vs Western blotting) and resolving experimental conditions (e.g., using the right mix of detergents in the case of UIC2) will, more often than not, overcome this obstacle.
8. Characteristically, several commercially available anti-Pgp mAbs, including mAb C219 and the widely used mAb JSB-1, have proven to exhibit cross-reactivity with c-ERB2 ***(10, 11)*** and pyruvate carboxylase ***(12)***, respectively. To avoid this pitfall, UIC2 epitope specificity was extensively characterized and subsequently internally validated for each intended application.
9. It was the multifaceted UIC2 characterization, using methodologies and detection systems providing direct answers to experimental questions at hand, that allowed for successful commercialization of the UIC2 mAb and its high efficiency in end-use applications. Same logic applies to the development of therapeutic mAbs.
10. Continuous monitoring of mAb-producing cultures and, if needed, recloning are required to maintain stable high levels of mAb production and avert cross-contaminations by extraneous hybridomas. Long-term passaging of initial hybridoma cultures should be avoided to prevent overgrowth by non-producing clones. Frequent freezing and short (weeks at a time) manufacturing cycles will help to obviate these problems.
11. Loss of mAb activity sometimes occurs as a result of mAb storage and transportation, especially during summer time when packages are exposed to higher temperatures both in the plane and on the ground.
12. The described low-cost procedures can be used to produce large amounts of mAbs at practically any academic lab or core facility. The key to large-scale, low-cost manufacturing is having a hybridoma that had been selected for growth in serum-free, protein-free media and for efficient production of the mAb. In the case of UIC2, 10 mg of the mAb could be readily harvested from tissue culture supernatants from just one 24-well plate. Scaling up to gram levels was also achievable using relatively inexpensive plastic cell factories or roller bottles.
13. The right choice of mAb presentation and, in particular, labeling for subsequent secondary detection are critical for efficient end-use applications. For example, labeling UIC2 with FITC produced weak and practically useless fluorescent conjugates for flow cytometry studies, while labeling

with Phycoerythrin resulted in reliable and consistent direct and indirect secondary detection. As discussed above, azide-free presentation of UIC2 was necessary for functional studies. Consequently, only commercial UIC2 preparations suitable for its end-use applications were prepared and marketed by UIC2 mAb suppliers.

14. Examples of inaccurate and simply incorrect information provided by commercial mAb sources are many and diverse, and can be a challenging subject for a separate paper. In general, the researcher should be always vigilant and must always cross-reference mAb information provided by the company with the original publications, subsequent studies, and other publicly available sources (e.g., vast NIH resources; www.pubmed.gov). Extensive internal validation of mAbs provided by different companies is also a must.
15. The ability to use mAb against human antigens in the rodent tissue environment is yet another advantage of utilizing transduced syngeneic mouse cells for immunization and selection. Equally importantly, resulting mAbs can be readily utilized in animal research and pre-clinical studies using mAbs with potential for use in therapeutic applications.
16. As discussed above, extensive internal validation of the mAb and related protocols is essential for its efficient utilization in end-use applications. For example, information for “prediluted” mAb preparations provided by some commercial sources is often incomplete and deceptive in that the mAb arrives in its final form and no internal validation is suggested. In this context, each and every new mAb preparation or protocol should be internally validated and cross-verified against previous mAb batches using the same detection technique.

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Recombinant Antibody Expression and Purification

Achim Knappik and Ralf Brundiers

1. Introduction

1.1. Recombinant Antibodies

Developments in the fields of bacterial expression of functional antibodies and methods to select genes from a library by using the phenotype of the encoded polypeptide have been a breakthrough in antibody technology. Today, phage display in combination with antibody gene libraries is widely used to select *E. coli* host cells that express desired antibody fragments. Such gene libraries are typically produced either from natural sources (e.g., from the spleen of an immunized animal or from plasma cells of human donors) or generated by genetic engineering. The latter has been used to create naïve libraries based on one or more antibody VH and VL gene segments that are diversified by cassette mutagenesis or similar approaches. Such libraries are typically unbiased and can be used for any given antigen ***(1–3)***. Modern naïve libraries are generally large (more than 10^{10} members), contain only few non-functional members, yield antibodies that are well expressed in *E. coli* (more than 1 mg of purified material per liter of culture) and are designed to allow further affinity maturation, if needed. Phage display is then most often used to select desired antibodies from such libraries (*see* ***refs. 4*** and ***5*** for reviews).

Recombinant antibodies offer many advantages over traditionally generated monoclonal antibodies that are only beginning to be explored. These advantages stem from two properties: the ability to assess the antibody DNA within an *E. coli* environment that allows the use of well-known genetic engineering methodologies, and the use of antibody fragments rather than intact IgG molecules, because of their smaller size and because of the absence of the Fc domain. The latter eliminates nonspecific binding to cellular Fc receptors and avoids other, sometimes unwanted, effects caused by the Fc part of intact IgG.

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1.2. *E. coli* Expression of Recombinant Antibodies

Since antibodies contain disulfide bonds that are necessary for maintaining their overall structure and binding activity, the folding of the molecule has to take place under oxidizing conditions. Gram-negative *E. coli* provides an oxidizing compartment between the cytoplasmic and outer membrane, the so-called periplasmic space. Hence, the attachment of bacterial leader sequences to the N-terminus of an antibody fragment directs the translated antibody chain(s) into the periplasmic space by the bacterial transport apparatus, where folding can take place under oxidizing conditions. To achieve secretion of antibody Fab fragments, both chains must be equipped with signal sequences. In order to cosecrete both chains to the bacterial periplasm, they are best arranged in an artificial operon, thereby being coordinately regulated by one promoter **(Fig. 1)**. In most current systems, the antibody light chain genes precedes the heavy chain gene segment in the two-cistron system, leading to higher expression levels of the light chain. This ensures that the amount of heavy chain without light chain partner is minimized, since unpartnered heavy chain is mostly insoluble, which

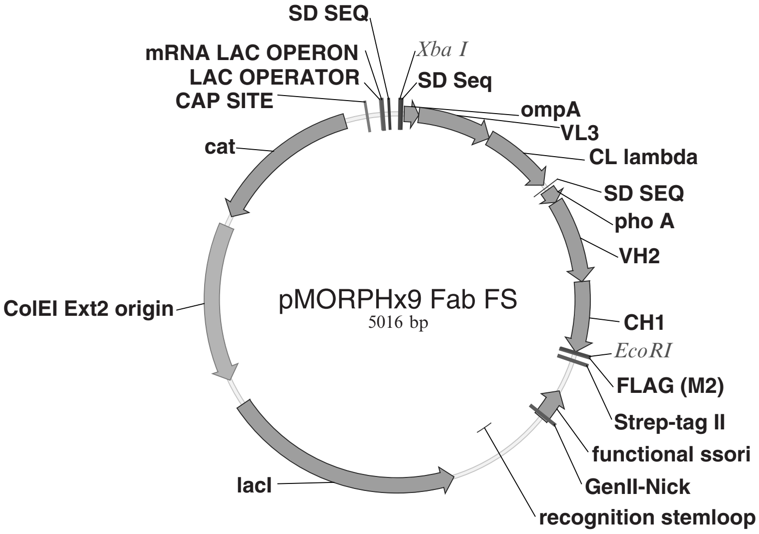
SD SEQ
mRNA LAC OPERON
LAC OPERATOR
CAP SITE
Xba I
SD Seq
ompA
VL3
CL lambda
SD SEQ
pho A
VH2
CH1
EcoRI
FLAG (M2)
Strep-tag II
functional ssori
GenII-Nick
recognition stemloop
cat
ColEI Ext2 origin
lacI
pMORPHx9 Fab FS
5016 bp

Fig. 1. Schematic view of an antibody Fab expression vector. Features are: a high-copy ColEI origin of replication, a chloramphenicol resistance gene (cat), a lac-promotor/operator region that is inducible by IPTG, and a lacI gene for repression of transcription when the inducer is absent. The Fab gene is arranged as bicistronic unit, flanked by the unique restriction sites XbaI and EcoRI. Each chain is equipped with signal sequences (ompA for the light chain and phoA for the heavy chain). Tags and other functional elements are encoded in frame after the EcoRI site, leading to fusions at the C-terminus of the CH1 domain. In the example shown here, the Fab is fused to a FLAG and and a StrepII tag.

induces a stress to the cell. Since purification tags are fused to the heavy chain (see below), excess light chain will not be purified.

The folding of periplasmic antibody protein does not proceed quantitatively for many antibody fragments. The yield of this process depends on external factors such as temperature, but also on the type of fragment and antibody primary sequence. Modern synthetic antibody libraries use stable and well-expressed antibody frameworks built form codon-optimized sequences, leading to yields of purified active material well above 1 mg per L of standard shaking flask culture **(Fig. 2)**. Such expression yields are sufficient to allow screening of antibody expressing clones after library selection by cultivation in 384 well microtiter plates. After induction of antibody expression, E. coli cells are subsequently lysed (either gently by an osmotic shock procedure to release the periplasmic content, or simply by adding lysozyme). The antibody containing lysates can then be tested by ELISA or other methods for the presence of antigen-specific antibody material. Screening by ELISA is one of the most used and well-established methods to screen antibody expressing clones. Target molecules can be directly coated onto 384-well microtiter plates, or biotinylated target antigens can be captured onto streptavidin plates. Bacterial extracts are added to the micro titer plates and bound antibody fragments are detected with enzyme-conjugated polyclonal antiserum or with antibodies directed against

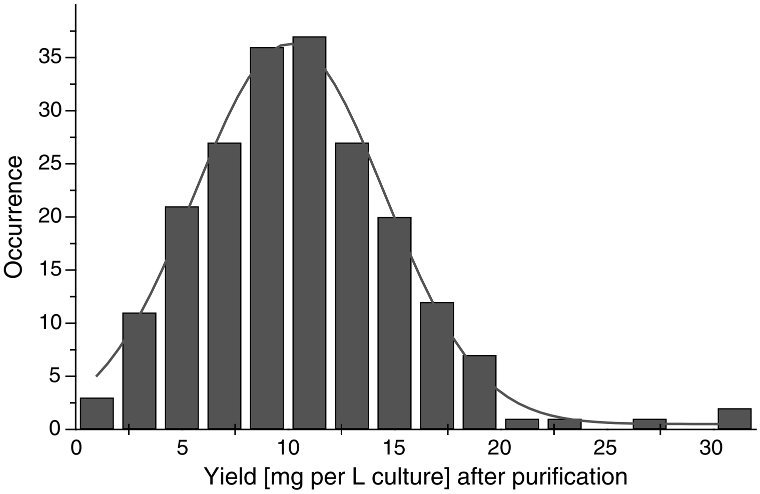

Fig. 2. Histogramm of expression yields after purification from 206 independent clones obtained from the HuCAL® library after phage display selections. Antibodies have been expressed either in monovalent or bivalent Fab format and purified using one-step NiNTA affinity chromatography. Expression yields follow an approximate Gaussian distribution with a center at 10 mg per liter culture volume.

the C-terminal tags provided by the vector (see **Fig. 1**). Often positive hits are sequenced to determine the number of unique antibodies from a selection. The process described above can be automated to a large extent using standard pipetting robots, thereby enabling a massive increase in throughput ***(6)***, one of the advantages over animal-based methodologies. Screening of recombinant antibody fragments on whole cells ***(7)*** and tissues ***(8)*** have also been reported.

After screening, the antibodies can be individually expressed and purified in larger scale. Soluble antibody fragments produced by bacterial colonies isolated as explained above are typically purified by one-step affinity chromatography using peptide tags that have been fused to the C-terminus of the antibody fragment. After purification, the material can be mostly aliquoted and stored frozen, similar to traditional monoclonal antibodies.

1.3. Antibody Formats

The two major types of recombinant antibody fragments that are usually expressed in *E. coli* are named single-chain Fv (scFv) and Fab.

By introducing a short sequence encoding a peptide linker between the VH and the VL gene segments a single polypeptide chain is created that folds into the so-called scFv antibody fragment ***(9)***. A variety of linkers has been tested, and the most frequently used one is a repetition of the sequence Gly_4Ser with 3 or 4 units. It has been shown that shortening the linker between the two variable domains leads to so-called diabodies ***(10)***, which pair with the complementary domains of another scFv and thereby promote the assembly of dimeric or bispecific molecules with two functional antigen binding sites. Since scFv is encoded by a single gene, expression systems do not need coordinated expression of two chains as with the heterodimeric Fab molecule. In addition, in vitro display systems, such as ribosome display ***(11)*** need single polypeptide chains as library members, in order to avoid swapping of chains and thereby loss of the phenotype – genotype linkage.

Another commonly used recombinant antibody fragment is the Fab fragment (**Fig. 3**), which is composed of the truncated heavy chain containing the variable and the first constant region (the so-called Fd fragment), and the entire light chain composed of the variable and constant domain. These two polypeptides are either covalently linked by disulfide bridges at the C-terminus, or are produced in higher yields without those, which nevertheless lead to highly stable H/L heterodimers ***(7)***. Advantages of the Fab fragment are (i), the similarity with the full-length Ig molecule, since it does not contain an artificial linker sequence which might interfere with the antigen binding site, (ii), the fact that well-established anti-Fab detection antibodies can be used and (iii), the fact that it is truly monovalent (which is not always the case with scFv fragments). The latter property is important for example when avidity effects should be avoided, e.g. when the intrinsic binding affinity needs to be determined.

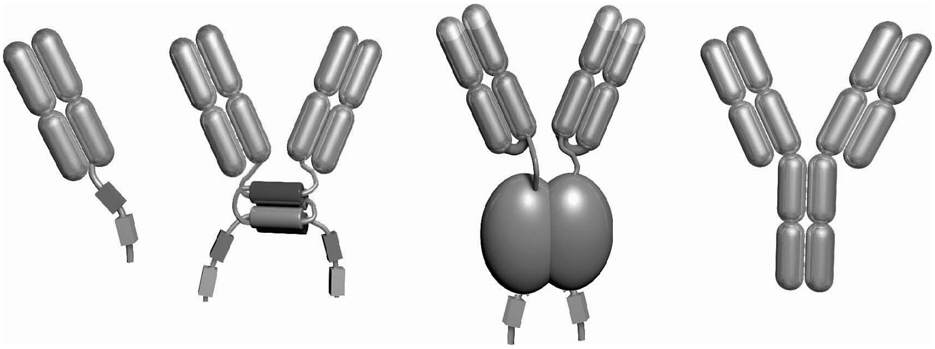

Fig. 3. Comparative schematic view of monovalent Fab with two peptide tags, bivalent Fab (dimerized by a helix-turn-helix motif) with two peptide tags, Fab fused to bacterial alkaline phosphatase (BAP) with one peptide tag, and IgG1. The region involved in antigen binding is shown in golden color. Note that the BAP fusion leads to bivalent Fab molecules, since BAP is a homodimer.

Antibody fragments usually have the same antigen-binding specificity as the corresponding intact antibody, since the complete antigen binding site is present. However, multivalency, which is a very effective means of increasing the functional affinity (avidity) to a surface-bound or polymeric antigen, is a very general property of antibodies. IgG contain two binding sites per molecule, which increases the apparent affinity (avidity) compared to a Fab or monomeric scFv antibody fragment. The most noticeably example is IgM, which carries 10 recognition binding sites. For particular applications, multivalency might be advantageous or even required. Thus, bivalency and further multivalency have been also engineering for recombinant antibody fragments. ScFv or Fab fragments can be dimerized by linking them to a small modular dimerization domain in the form of one or two amphipatic helices *(12)*. For example, the leucine zipper from the yeast transcription factor GCN4 has been shown to be suitable as a dimerization device. These "miniantibodies" assemble in dimeric form in E. coli with typically no loss in final yield, and the binding performance in assays like IHC or Western blot is mostly indistinguishable from a whole antibody in avidity. Antibody fragments fused to the small tetramerization domain of p53 can form tetrameric molecules *(13)*.

Genetic fusions to enzymes like bacterial alkaline phosphatase (BAP) connect the binding and detection capability into one molecule (*see* **Fig. 3**). Even such fusions, which lead to molecules of the size of full-length IgG antibodies (about 150 kDa) can be expressed in *E. coli* with almost the same yields as smaller antibody fragments. Since the BAP is a homo-dimer, the resulting molecules are bivalent. Many other antibody fusions for various applications have been described in the literature, such as core streptavidin for avidity increase, beta-lactamase for prodrug activation or interleukin-8 fusion for neutrophil activation, to name a few. Clearly this field is still in its infancy.

1.4. Peptide Tags for Purification and Detection

Purification of whole antibodies has mostly relied on antigen-affinity chromatography or affinity chromatography using bacterial immunoglobulin-binding proteins such as proteins A and G. However, the usefulness of this strategy for antibody fragments is limited, as these bacterial proteins bind mostly to the constant domains. However, the generation of antibodies *in vitro* enables manipulation of their sequences, for instance by linking desired sequences such as affinity tails to the antibody framework regions. Such affinity tails are often short peptide tags, which are used for purification, but also for antibody immobilization and detection. Typically, such fusions are cloned in-frame at the 3′-end of the antibody gene, leading to a maximum distance in the native fusion protein between the antigen-binding site and the additional functionality. A few such peptide fusions will be highlighted here:

Peptide tags are mostly used for affinity purification purposes. Most tags that have been generally developed for recombinant protein purification will

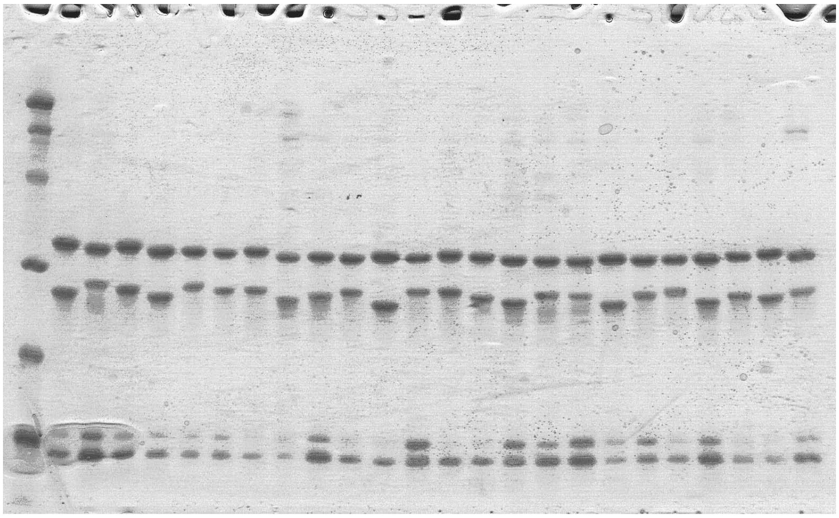

Fig. 4. Coomassie-stained SDS gel (15%, Criterion gel, Bio-Rad, low range marker, Bio-Rad) of 24 different His-tagged Fab antibodies after NiNTA purification, all in the bivalent format. The amount of 1.5 µg total protein was loaded on each lane. The heavy chain (equipped with a helix-turn-helix motif and the myc- and his$_6$-peptide tags) runs at about 35 kDa, whereas the light chain typically runs below 30 kDa. The impurities at about 15 kDa are C-terminal CH1 fragments that contain the His$_6$-tag and therefore co-purifiy. They do not contain any aromatic amino acids and therefore do not interfere with A280nm measurements.

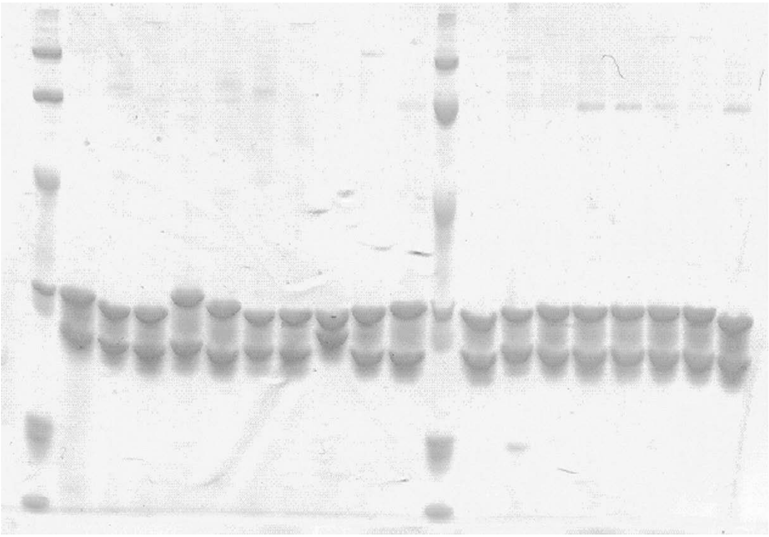

Fig. 5. Coomassie-stained SDS gel (15%, Criterion gel, Bio-Rad, low range marker, Bio-Rad) of 18 different Strep-tagged Fab antibodies after Strep-tag purification. The amount of 3 µg total protein was loaded on each lane. The heavy chain runs at about 31 kDa, whereas the light chain typically runs below 30 kDa. In comparison to the NiNTA purification, the Strep-tag purification combined with the periplasmic preparation will result in significant higher purities (usually >90%).

also work for antibodies. The most convenient strategy is probably the use of a stretch of histidines, the so-called his-tag, a series of 5 to 6 histidines that bind to affinity media such as NTA-agarose or Talon resin, when metal ions (nickel or cobalt) are bound. His-tagged proteins bind with milli-molar affinity to the column and are gently eluted with 150–300 mM imidazole **(Fig. 4)**. Another such tag is the StrepII-tag ***(14)***, which shows affinity to streptavidin- or streptactin-sepharose (streptactin is a genetically engineered streptavidin with higher affinity to the Strep-tag) **(Fig. 5)**. Commercial antibodies to both tags are available, so the tags can be used for detection purposes as well. Other detection tags like the V5-tag (GKPIPNPLLGLDST), which is derived from a small epitope present on the P/V proteins of the paramyxovirus, SV5, or the myc-tag (EQKLISEEDL), which correspond to residues 408–439 of the human p62 c-myc protein are also frequently used, because specific high-affinity monoclonal antibodies are commercially available. The FLAG-tag (DYKDDDDK) is of special interest since a monoclonal antibody (termed M1) exists that only binds to the tag when the N-terminus is free and not involved in a peptide bond. This has been used to monitor cleavage of signal sequences during *E. coli* antibody expression ***(15)***.

2. Materials

2.1. Bacterial Cell Culture

1. 2xYT Medium:
   - 2xYT (Yeast Extract Tryptone) Media; Difco.
   - Dissolve 155 g 2xYT powder in 5 L deionized water.
   - After total dissolution, autoclave at 121°C for 20 min.
   - Store at room temperature.
2. Glucose stock (40%):
   - D(+)-glucose monohydrate, (MW = 198 g/mol).
   - Dissolve 44 g glucose in 100 mL deionized water.
   - After total dissolution, sterile filtration using 0.2 µm filter.
   - Store at room temperature.
3. CAM (Chloramphenicol) stock (34 mg/ml in ethanol):
   - Dissolve 3.4 g chloramphenicol in 100 mL ethanol (p.a.).
   - After total dissolution, sterile filtration using 0.2 µm filter.
   - Store at −20°C.
4. IPTG stock (1M in ddH_2O):
   - Dissolve 4.2 g IPTG in 100 ml deionized water.
   - After total dissolution, sterile filtration using 0.2 µm filter.
   - Store at −20°C.
5. Preculture medium: mix 974 ml 2xYT medium with 25 ml glucose stock solution and 1 ml CAM-stock solution.
6. Expression-culture medium: mix 974 ml 2x YT-medium with 2.5 ml glucose stock solution and 1 ml CAM-stock solution.
7. Sterile filter: Acrodisc 13 mm syringe filters, 0.2 µm (Pall).

2.2. Cell Lysis

1. Cell Lysis Buffer: Take 120 ml BugBuster (Merck), add 240 mg Lysozyme (Roche), 1250 U Benzonase (Merck; 250 U/µl = 5 A/µl), and 5 PIT (Complete Protease Inhibitor) tablets, EDTA free (Roche #1873580) (see **Note 1**).
   Always prepare fresh solution.
2. BSS buffer: 200 mM boric acide, 160 mM NaCl, pH 8.0. Dissolve 12.37 g boric acid add 9.35g NaCl in 1000ml deionized water. Adjust to pH to 8.0 with NaOH. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
3. PeriPrep lysis buffer: 200 mM boric acide, 160 mM NaCl, 2 mM EDTA, protease Inhibitor, pH 8.0. Dissolve 0.75g EDTA: Titriplex®III, (VWR, #108421) and 40 PIT (Complete Protease Inhibitor) tablets, EDTA free, (Roche), in 1000 ml BSS buffer (*see* **Note 1**). Adjust to pH to 8.0 with NaOH.
   Always prepare fresh solution.
4. Avidin stock solution 10mg/ml (for Strep-Tag purifications) (*see* **Note 2**). Dissolve 10 mg avidin in 10ml deionized water. Store at −20°C.
5. Seriflip filter device (STERIFLIP, Milipore).

2.3. Affinity Chromatography

2.3.1. IMAC Affinity Chromatography

1. IMAC-RB (running buffer): 20 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazol, pH 7.4. Dissolve 2.4 g NaH_2PO_4, 29.2 g NaCl, 0.68g imidazole in 1000ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
2. IMAC-WB (washing buffer): 20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazol, pH 7.4. Dissolve 2.4 g NaH_2PO_4, 29.2 g NaCl, 1.36 g imidazol in 1000 ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
3. IMAC-EB (elution buffer): 20 mM NaH_2PO_4, 500 mM NaCl, 250 mM Imidazole, pH 7.4. Dissolve 2.4 g NaH_2PO_4, 29.2 g NaCl, 17 g imidazole in 1000 ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature (*see* **Note 3**).
5. Ni-NTA agarose (Qiagen) (*see* **Note 4**).
4. Gravity flow plastic columns: Poly Prep (BioRad).

2.3.2. Strep Tag Affinity Chromatography

1. Strep-RB (running buffer): 100 mM Tris-HCl, 750 mM NaCl, 1 mM EDTA, pH 8.0. Dissolve 12.1 g Tris, 43.8 NaCl, 0.37g EDTA in 1000 ml deionized water. Adjust to pH to 8.0. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
2. Strep-EB (elution buffer): Strep-RB + 5 mM D-Desthiobiotin. 100 mM Tris-HCl, 750 mM NaCl, 1 mM EDTA, 5 mM D-desthiobiotin, pH 8.0. Dissolve 12.1 g Tris, 43.8g NaCl, 0.37 g EDTA, 1.1 g D-D-desthiobiotin in 1000 ml deionized water. Adjust to pH to 8.0. After total dissolution, sterile filtration using 0.2 µm filter. Store at 4°C and not longer than 1 week (*see* **Note 5**).
3. Strep-RegB (regeneration buffer): 5 mM HABA, 100 mM Tris-HCl, pH 8.0. Dissolve 1.21 g HABA (HABA-ImmunoPure Pierce/Perbio) and 12.1 g Tris in 1000ml deionized water. Adjust to pH to 8.0 with HCl. After total dissolution, sterile filtration using 0.2 µm filter. Store at 4°C.
4. Streptactin sepharose (IBA-GMBH).
5. Gravity flow plastic columns: Poly prep (BioRad).

2.3.3. Size Exclusion Chromatography

1. PBS: 0.136 M NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4. Dissolve 0.2g KCl, 8g NaCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 in 1000ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
2. 3xPBS: 0.401 M NaCl, 8.04 mM KCl, 24.3 mM Na_2HPO_4, 4.38 mM KH_2PO_4, pH 7.4. Dissolve 0.6 g KCl, 24 g NaCl, 4.32 g Na_2HPO_4, and 0.72g KH_2PO_4 in 1000 ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.

3. PD10 columns: GE (Amersham Pharmacia Biotech).

2.3.4. Sterile Filtration, Concentration Determination, SDS-PAGE

1. Sterile filter: Acrodisc 13mm syringe filters, 0.2 µm (Pall).
2. UV-compatible cuvette: UVette (VWR international).
3. SDS-sample buffer 5 x concentrated:
   125 mM Tris-HCl pH6,8 10% glycerol, 0,5% SDS, 0,05% (w/v) bromophenol blue, 5% beta-mercaptoethanol.
4. SDS running buffer: 25 mM Tris, 192 mM glycine, 0,1% SDS.
5. SDS-PAGE system: Criterion 15% Tris. 26W (Bio-Rad).
6. Gel staining solution: Gelcode Blue stain reagent (Pierce).

3. Methods

3.1. Strep-Tag Expression and Purification

3.1.1. Preculture

1. Fill 20 ml medium 1 (2xYT + 1% glucose + CAM) into a 100 ml Erlenmeyer flask.
2. Inoculate culture with a single colony with a sterile inoculating loop. Freshly transformed TG1F$^-$ cells are recommended (*see* **Note 6**).
3. Incubate at 30°C, O/N (18 h +/– 2h), 250 rpm.

3.1.2. Expression Culture

1. Fill 750 ml medium 2 (2xYT + 0.1% glucose + CAM) into a 2 L baffled Erlenmeyer flask.
2. Inoculate with 1/300 volume (2.5 ml) of preculture.
3. Incubate at 30°C, 3 h, 180 rpm.
4. Induce with 560 µL IPTG (1M) stock solution (0.75 mM final concentration).
5. Express Fab at 30°C, 160 rpm, O/N (20 h +/–2 h post induction time) (*see* **Note 7**).

3.1.3. Cell Harvest and Periplasmic Prep (PP)

1. Harvest cells by centrifugation at 5000 g, 30 min, 4°C.
2. Decant supernatant and discard after autoclaving (*see* **Note 8**).
3. Resuspend the bacterial pellet with 30–35 ml cold (<10°C) PeriPrep lysis buffer by pipetting carefully up and down or by shaking at 250 rpm.
4. Transfer suspension into 50 ml Falcon tube and adjust volume to 40 ml.
5. Mix suspension carefully by inverting the tube several times.
6. Incubate O/N, 4°C.

3.1.4. Purification

1. Centrifuge suspension at 16000 g, 4°C, 30 min.
2. Transfer supernatant into a fresh 50 ml Falcon tube by decanting.

3. Add 50 µL avidin stock per sample.
4. Incubate 30 min, 4°C.
5. Filter supernatant with Steriflip filtration device (0.22µm) (*see* **Note 9**).
6. Load a Poly-Prep column with 2 ml Streptactin sepharose (50%; 1 ml bed volume).
7. Equilibrate column with 30 ml Strep-RB by filling once the funnel (~30ml).
8. Load sample to the column and collect the flow through in the respective Falcon tube.
9. Remove funnel and wash twice with 5 ml Strep-RB.
10. Add 0.5 ml Strep-EB and discard flow through.
11. Place column on 2 ml Eppendorf cup.
12. Elute Fab with 2 ml Strep-EB and collect eluate; continue with step buffer exchange.

3.1.5. Regeneration of Strep-Tactin Columns (Optional)

1. After elution regenerate column two times with 5 ml Strep-RegB.
2. Place a funnel onto the column and re-equilibrate the column with 30 ml Strep-RB.
3. Store columns in approx. 2 ml Strep-RB at 4°C (*see* **Note 10**).

3.2. His-Tag Expression and Purification

3.2.1. Preculture

1. Fill 20 ml medium 1 (2×YT + 1% glucose + CAM) into a 100 ml Erlenmeyer flask.
2. Inoculate culture with a single colony with a sterile inoculating loop. Freshly transformed TG1F$^-$ cells are recommended (*see* **Note 6**).
3. Incubate at 30°C, O/N (18 h +/−2 h), 250 rpm.

3.2.2. Expression Culture

1. Fill 750 ml Medium 2 (2 × YT + 0.1% glucose + CAM) into a 2 L baffled Erlenmeyer flask.
2. Inoculate with 1/300 volume (2.5 ml) of preculture.
3. Incubate at 30°C, 3 h, 180 rpm.
4. Induce with 560 µL IPTG (1M) stock solution (0.75 mM final concentration).
5. Express Fab at 30°C, 160 rpm, O/N (20 h +/− 2 h post induction time) (*see* **Note 7**).

3.2.3. Cell Harvest and Whole Cell Lysis (*see* **Note 11**)

1. Harvest cells by centrifugation at 5000 g, 30 min, 4°C.
2. Decant supernatant and discard after autoclaving (*see* **Note 8**).
3. Resuspend the bacterial pellet with 10 ml cold whole-cell lysis buffer by pipetting carefully up and down or by shaking at 250 rpm.
4. Transfer suspension into 50 ml Falcon tube and mix suspension carefully by inverting the tube several times.
5. Incubate for 30 min, at room temperature.
6. Centrifuge suspension at 16000 g, 4°C, 30 min.

7. Transfer the cleared supernatant in a new Falcon tube.
8. Filter supernatant with Steriflip filtration device (0.22 µm) (*see* **Note 9**).

3.2.4. Purification

1. Load a Poly-Prep column with 2 ml Ni-NTA-Sepharose (50%; 1 ml bed volume).
2. Equilibrate column with IMAC-RB by filling once the funnel (~30 ml).
3. Load sample to the column and collect the flow through in the respective Falcon tube.
4. Remove funnel and wash three times with 5 ml IMAC-WB.
5. Add 0.5 ml IMAC-EB and discard flow through (*see* **Note 4**).
6. Place column on 2 ml Eppendorf cup.
7. Elute Fab with 2 ml IMAC-EB and collect eluate; continue with step buffer exchange.

3.3. SEC for Buffer Exchange

3.3.1. Buffer Exchange with PD10 Desalting Column

1. Place column in a rack (e.g. Biorad polycolumn rack) and equilibrate the column with 20 ml PBS (for Fab) or 3xPBS (for bivalent Fab constructs) (*see* **Note 12**).
2. After running buffer through the column, add a total volume of 2 ml of the purified Fab solution.
3. Discard the eluent.
4. When the sample has run into the column, add additional 1 ml of PBS (or 3xPBS).
5. Discard the eluent.
6. When the buffer has run into the column, place a 15 ml Falcon tube under the column and elute the purified Fab with 2.5 ml PBS (or 3xPBS).
7. Wash the column with 20 ml PBS (or 3xPBS).
8. Store columns in approx. 2 ml PBS (or 3xPBS).
   Columns can be reused several times.

3.3.2. Sterile Filtration and Concentration Determination

1. Filter eluate with 0.2 µm filter (Pall; 13 mm) fixed to 2 ml syringe into fresh 15 ml Falcon tube.
2. Take an aliquot of 200 µL for **step 3.3.3**.
3. Fab samples can be stored under sterile condition for several weeks. For long term storage, freezing at –20°C or –80°C is recommended (*see* **Note 13**).

3.3.3. Determination of Concentration and Purity

1. Determine the protein concentration by measuring the absorption at 280 nm (*see* **Note 5**).
   A_{280}nm × 0.65 = concentration in mg/ml (for monovalent Fab constructs).
   A_{280}nm × 0.7 = concentration in mg/ml (for bivalent Fab constructs).
   (dilute sample 1:4 or 1:10 with PBS or (3xPBS) to get values in the linear range between (0.1 and 1AUF).
2. Take up to 5 µg of you protein sample and add with SDS-sample buffer and water to a final volume of 25 µl.

3. Heat sample for 5 min to 95°C for total denaturation.
4. Load protein-sample on 15% Tris-HCl SDS-PAGE (e.g., Biorad Criterion System). Protein amounts between 1.5 and 3 µg are recommended for optimal visual inspection of Fab-bands and impurities.
   Run SDS-PAGE according to manufacture instructions.

4. Notes

1. Use of protease inhibitor mix is not needed in general but recommended to avoid possible degradation of some Fab fragments. The by-products in the purification of bivalent Fab including the BAP fusion construct are not effected by the addition of the protease inhibitor. Be aware that some protease inhibitor mixes contain EDTA or EGTA. Don't use these ingredients in IMAC purification, the metal ion will be chelated and the material will loose binding-activity.
2. Avidin will bind traces of biotin produced by E. coli. In particular, if a Strep-tag column is used several times the biotin will poison the column because it has a much higher affinity to Streptactin as the Strep-tag. At this point washing with Strep-RegB is not sufficient to regenerate the column.
3. Elution of Fab fragments will start at imidazol concentrations of ~50 m*M*, the bivalent construct with two tags will elute at higher concentrations (>75 m*M*).
4. Ni-NTA shows good results in our hands, but some authors prefer other IMAC resins (e.g., Talon-resin or Zn/Cu ions).
5. Desthiobiotin takes a long time to dissolve (gentle warming helps). Low concentrations of desthiobiotin will not interfere with many assays. It also shows no significant UV-absorption or interference in protein concentration assays. The final product is often also very pure (>90% judged on SDS-page). So it is often possible omit buffer exchange and use the eluted product directly in many applications.
6. For optimal expression performance it could be helpful to screen several clones. About 80% of all clones from the HuCAL® library will express in a range of +/–20%. In some case it is possible to find clones which show much higher expression rates (*see* **Fig. 2**).
7. These conditions show the best results for most Fab fragments. Nevertheless it could be helpful to optimise the expression conditions for optimal results. In some cases, lower temperatures (25°C) and lower IPTG concentrations (0.2 mM) will give better results.
8. In some cases (depending on the individual antibody and on the expression conditions, e.g. long expression times, or media that contains glycerol), significant amounts of the Fab can be found in the expression media. This can be checked with an anti-Fab-ELISA.

9. Filtration can be omitted but this may cause clogging or contamination of the columns, in particular if the columns are used several times.
10. Streptactin columns can be used more than 10 times over more than 6 month without significant loss of binding activity.
11. For purification of bivalent Fab or large Fab-fusions it is not recommended to use a periplasmic preparation, because the release in the lysis buffer is often limited and the purification yields drop down.
12. The use of 3xPBS is recommended in purifications of bivalent Fab fragments, because these constructs sometimes tend to aggregate in PBS (depending on the individual antibody and the final concentration). This aggregation can be avoided by using a buffer with higher ionic strength like 3xPBS.
13. After thawing 3xPBS samples, a precipitate can often been seen at the bottom of the well or tube. This is salt from the PBS. It will disappear after thawing and gentle shaking.

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Screening Hybridoma Culture Supernatants Using Solid-Phase Radiobinding Assay

Mark Page and Robin Thorpe

1. Introduction

A large number of hybridoma culture supernatants (up to 200) need to be screened for antibodies at one time. The assay must be reliable so that it can accurately identify positive lines, and it must be relatively quick so that the positive lines, which are 75–100% confluent, can be fed and expanded as soon as possible after the assay results are known. Solid-phase binding assays are appropriate for this purpose and are commonly used for detection of antibodies directed against soluble antigens ***(1)***. The method involves immobilizing the antigen of choice onto a solid phase by electrostatic interaction between the protein and plastic support. Hybridoma supernatants are added to the solid phase (usually a 96-well format) in which positive antibodies bind to the antigen. Detection of the bound antibodies is then achieved by addition of an antimouse immunoglobulin labeled with radioactivity (usually ^{125}I) and the radioactivity counted in a γ counter.

2. Materials

1. Antigen: 0.5–5 µg/mL in phosphate-buffered saline (PBS) with 0.02% sodium azide.
2. PBS: 0.14 *M* NaCl, 2.7 m*M* KCl, 1.50 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4.
3. Blocking buffer: PBS containing either 5% v/v pig serum, 5% w/v dried milk powder, or 3% w/v hemoglobin (*see* **Note 1**).
4. Hybridoma culture supernatants.
5. Negative control: irrelevant supernatant or culture medium.
6. Positive control: serum from immunized mouse from which spleen for hybridoma production was derived.
7. Antimouse/rat immunoglobulin, ^{125}I-labeled (100 µCi/5 µg protein).

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3. Method

1. Pipet 50 µL of antigen solution into each well of a 96-well microtiter plate and incubate at 4°C overnight. Such plates can be stored at 4°C for several weeks. Seal plates with cling film to prevent the plates from drying out (*see* **Note 2**).
2. Remove antigen solution from wells by either a Pasteur pipet (cover the tip with a small piece of plastic tubing to prevent scratching the antigen-coated wells) or by shaking the contents from the plate in one quick movement (*see* **Note 3**).
3. Wash the plate by filling the wells with PBS and rapidly discarding the contents. Repeat this twice more, tap the plate dry, then fill the wells with blocking buffer, and incubate at room temperature for 30 min to 1 h. Wash three more times with PBS.
4. Pipet 100 µL of neat hybridoma supernatant into the wells, and incubate for approx 3 h at room temperature. Include the negative and positive controls.
5. Wash with blocking buffer three times.
6. Dilute the ^{125}I-labeled antimouse immunoglobulin in blocking buffer to give 1×10^6 cpm/mL. Add 100% µL to each well, and incubate for 1 h at room temperature.
7. Wash with blocking buffer.
8. Cut out individual wells using scissors or a hot nichrome wire plate cutter, and determine the radioactivity bound using a γ counter. A positive result would have counts that are four to five times greater than the background. Normally, the background counts would be around 100–200 cpm with a positive value of at least 1000 cpm (*see* **Note 4**).

4. Notes

1. Filter the milk and hemoglobin solutions coarsely through an absorbent cloth (e.g., Kimnet, Kimberley Clark) to remove lumps of undissolved powder.
2. Antigens (diluted in distilled water) can be dried onto the plates by incubation in a warm room (37°C) without sealing. This method is usually preferred if the antigen is a peptide improving binding to the plastic; however, the background signal may be increased.
3. The antigen solution may be reused several times to coat additional plates, since only a small proportion of the protein adheres to the plastic.
4. Counting radioactivity in a 96-well format can also be performed by using plates designed for use in scintillation counters. The assay is performed as described, except that in the final step, a scintillation fluid (designed to scintillate with ^{125}I, such as Microscint 20, Packard Instrument Co.) is added to each well, and the scintillation counted in a purpose-built machine (e.g., Topcount, Packard Instrument Co.).

Reference

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Screening Hybridoma Culture Supernatants Using ELISA

Mark Page and Robin Thorpe

1. Introduction

Enzyme-linked immunosorbent assay (ELISA) is a widely used method for the detection of antibody and is appropriate for use for screening hybridoma supernatants ***(1)***. As with radiobinding assays, it is a solid-phase binding assay that is quick, reliable, and accurate. The method is often preferred to radioactive assays, since the handling and disposal of radioisotopes is avoided and the enzyme conjugates are more stable than radioiodinated proteins. However, most of the substrates for the enzyme reactions are carcinogenic or toxic and, hence, require handling with care.

Enzymes are selected that show simple kinetics, and can be assayed by a simple procedure (normally spectrophotometric). Cheapness, availability, and stability of substrate are also important considerations. For these reasons, the most commonly used enzymes are alkaline phosphatase, β-D-galactosidase, and horseradish peroxidase.

2. Materials

1. PBS-Tween: Phosphate-buffered saline (PBS) (0.14 *M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4) containing 0.05% Tween-20.
2. Antigen: 1–5 µg/mL in PBS.
3. Blocking buffers: PBS-Tween containing either 5% v/v pig serum or 5% w/v dried milk powder (*see* **Note 1**).
4. Hybridoma culture supernatants.
5. Alkaline phosphatase conjugated antimouse immunoglobulin.
6. Carbonate buffer: 0.05 *M* sodium carbonate, pH 9.6.
7. *p*-Nitrophenyl phosphate, disodium hexahydrate: 1 mg/mL in carbonate buffer containing 0.5 m*M* magnesium chloride.
8. 1 *M* sodium hydroxide.

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3. Method

1. Pipet 50 µL of antigen solution into each well of a 96-well plate, and incubate overnight at 4°C. Such plates can be stored for 4°C for several weeks. Seal plates with cling film to prevent the plates from drying out (*see* **Note 2**).
2. Remove antigen solution from wells by either a Pasteur pipet (cover the tip with a small piece of plastic tubing to prevent scratching the antigen-coated wells) or by shaking the contents from the plate in one quick movement (*see* **Note 3**).
3. Wash the plate by filling the wells with PBS and rapidly discarding the contents. Repeat this twice more, then fill the wells with blocking buffer, and incubate at room temperature for 30 min to 1 h. Wash three more times with PBS.
4. Pipet 100 µL of neat hybridoma supernatant into the wells, and incubate for approx 3 h at room temperature. Include the negative and positive controls (*see* **Note 4**).
5. Wash with blocking buffer three times.
6. Prepare 1:1000 dilution of alkaline phosphatase-conjugated antimouse immunoglobulin in PBS-Tween, and add 200 µL of this to each well. Cover, and incubate at room temperature for 2 h.
7. Shake-off conjugate into sink and wash three times with PBS.
8. Add 200 µL *p*-nitrophenyl phosphate solution to each well, and incubate at room temperature for 20–30 min.
9. Add 50 µL sodium hydroxide to each well, mix, and read the absorbance of each well at 405 nm (*see* **Note 5**). Typical background readings for absorbance should be less than 0.2 with positive readings usually three or more standard deviations above the average background value.

4. Notes

1. Filter the milk solution coarsely through an absorbent cloth (e.g., Kimnet, Kimberley Clark) to remove lumps of undissolved powder.
2. Antigens (diluted in distilled water) can be dried onto the plates by incubation in a warm room (37°C) without sealing. This method is usually preferred if the antigen is a peptide improving binding to the plastic; however, the background signal may be increased.
3. The antigen solution may be reused several times to coat additional plates, since only a small proportion of the protein adheres to the plastic.
4. All assays should be carried out in duplicate or triplicate.
5. Purpose-built plate readers provide a very rapid and convenient means of determining the absorbance.

Reference

1. Johnstone, A. and Thorpe, R. (1996) *Immunochemistry in Practice,* 3rd ed. Blackwell Science, Oxford, UK.

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Growth and Purification of Murine Monoclonal Antibodies

Mark Page and Robin Thorpe

1. Introduction

Once a hybridoma line has been selected and cloned, it can be expanded and seed stocks cryopreserved for future use. Relatively large amounts of purified MAb may also be required. There are a variety of procedures for this that ensure the establishment of a stable cell line secreting high levels of specific immunoglobulin. High concentrations of antibody can be generated by growing the line in the peritoneal cavity of mice/rats of the same strain as the tumor cell line donor and spleen cell donor. Antibody is secreted into the ascitic fluid formed within the cavity at a concentration up to 10 mg/mL. However, the ascites will contain immunoglobulins derived from the recipient animal that can be removed by affinity chromatography if desired. Several in vitro culture methods using hollow fibres or dialysis tubing ***(1)*** have been developed and are commercially available; this avoids the use of recipient mice/rats and contamination by host immunoglobulins, although contamination with culture medium-derived proteins may be a problem.

2. Materials

1. RPMI-1640/DMEM medium.
2. Fetal bovine serum (FBS).
3. Phosphate-buffered saline (PBS): 0.14 *M* NaCl, 2.7 m*M* KCl, 1.50 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4.
4. HT medium: 1 mL 50X HT supplement in 50 mL 10% FBS/RPMI.
5. HT supplement (Gibco, Paisley, UK).

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3. Method

3.1. Growth of Hybridomas

1. After cloning, grow hybridomas in 1 mL HT medium in separate wells of a 24-well plate. Check growth every day and feed with 0.5–1 mL HT medium if supernatant turns yellow (*see* **Note 1**).
2. When cells are 75–100% confluent (*see* **Note 2**), expand into two further wells with fresh HT medium. When these are confluent, transfer into a 25-cm^3 flask and feed with approx 10 mL medium (*see* **Note 3**). Subsequently, cell lines should be weaned off HT medium by increasing the dilution of HT medium stepwise with normal medium lacking the HT supplement, eventually culturing the cells in normal medium. Do this by reducing the amount of HT medium by 25–50% at each feed for expansion of the hybridomas. For example, reduce the amount of HT from 100 to 75 to 50 to 25 to 0% replacing with normal medium.
3. The FBS may be reduced to 5% or lower if hybridoma growth is strong.

3.2. Purification of MAb

MAb purification can be carried out by a number of methods as described in Chapters 179–190 and 207.

4. Notes

1. Phenol red is included in the medium as a visual indicator of pH. A yellow supernatant indicates acid conditions as a result of dissolved CO_2 (carbonic acid) derived from active cell growth (respiration). Normally, a yellow supernatant will indicate that the cells should be split (passaged) because the active cell growth will have exhausted the medium of nutrients and hence will not sustain cell viability.
2. Cells are confluent when the bottom of the flask/well is completely covered. Usually, the cells will require feeding or expanding at this stage (*see* **Note 1**).
3. To prevent inadvertant loss of precious cell lines, it may be prudent to freeze cloned lines before expansion into flasks.

Reference

1. Pannell, R. and Milstein, C. (1992) An oscillating bubble chamber for laboratory scale production of MAb as an alternative to ascitic tumors. *J. Immunol. Meth.* **146**, 43–48.

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Affinity Purification Techniques for Monoclonal Antibodies

Alexander Schwarz

1. Introduction

Monoclonal antibodies have many applications in biotechnology such as immuno-affinity chromatography, immunodiagnostics, immunotherapy, drug targeting, biosensors, and many others. For all these purposes homogeneous antibody preparation are needed. Affinity chromatography, which relies on the specific interaction between an immobilized ligand and a particular molecule sought to be purified, is a well-known technique to purify proteins from solution. Three different affinity techniques for the one-step purification of antibodies—protein A, thiophilic adsorption, and immobilized metal affinity chromatography—are described in this chapter.

1.1. Protein A Chromatography

Protein A is a cell wall component of *Staphylococcus aureas*. Protein A consists of a single polypeptide chain in the form of a cylinder, which contains five highly homologous antibody binding domains. The binding site for protein A is located on the Fc portion of antibodies of the immunoglobulin G (IgG) class ***(1)***. Binding occurs through an induced hydrophobic fit and is promoted by addition of salts such as sodium citrate or sodium sulfate. At the center of the Fc binding site as well as on protein A reside histidine residues. At alkaline pH, these residues are uncharged and hydrophobic, strengthening the interaction between protein A and the antibody. As the pH is shifted to acidic values, these residues become charged and repel each other. Differences in the pH-dependent elution properties (**Table 1**) are seen between antibodies from different classes as well as different species due to minor differences in the binding sites. These

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Table 1
Affinity of Protein A for IgG for Different Species and Subclasses

IgG species/subclass	Affinity	Binding pH	Elution pH
Human IgG1	High	7.5	3
Human IgG2	High	7.5	3
Human IgG3	Moderate	8	4–5
Human IgG4	High	7.5	3
Mouse IgG1	Low	8.5	5–6
Mouse IgG2a	Moderate	8	4–5
Mouse IgG2b	High	7.5	3
Mouse IgG3	Moderate	8	4–5
Rat IgG1	Low	8.5	5–6
Rat IgG2a	None–low		
Rat IgG2b	Low	8.5	5–6

differences can be successfully exploited in the separation of contaminating bovine IgG from mouse IgG, as shown in **Subheading 1.3**.

The major attraction in using protein A is its simplicity. The supernatant is adsorbed onto a protein A gel, the gel is washed, and the antibody is eluted at an acidic pH. The antibody recovered has a purity of >90%, often with full recovery of biological activity. The method described in **Subheading 3.1**. provides a more detailed description of the purification for high-affinity antibodies.

The affinity between protein A and the antibody is due mainly to hydrophobic interactions at the binding sites *(1)*. The interaction can be strengthened by the inclusion of higher concentrations of chaotropic salts such as sodium citrate or sodium sulfate. Although the addition of chaotropic salts is unnecessary in the case of high-affinity antibodies, it allows weakly binding antibodies such as mouse IgG1 to strongly interact with protein A. This purification scheme is outlined in **Subheading 3.2**. The different affinities resulting from minor differences in the Fc region can also be utilized for the separation of subclasses or the separation of different species in hybridoma supernatant using a pH gradient. However, this is possible only if the difference in affinity is large enough like in the case of the separation of mouse IgG2b from bovine IgG. If fetal calf serum supplemented growth media is used, bovine IgG will contaminate the monoclonal antibody if the method described in **Subheading 3.1**. or **3.2**. is used. An improved protocol utilizing the different affinities is described in **Subheading 3.3**.

1.2. Thiophilic Adsorption Chromatography

The term thiophilic adsorption chromatography was coined for gels, which contain low molecular weight sulfur-containing ligands such as divinyl sulfone structures or mercapto-heterocyclic structures ***(2,3)***. The precise binding mechanism of proteins to these gels is not well understood. However, thiophilic chromatography can be regarded as a variation of hydrophobic interaction chromatography inasmuch as chaotropic salts need to be added to facilitate binding of antibodies to the gel. Therefore, the interaction between the ligands and the antibody is likely to be mediated through accessible aromatic groups on the surface of the antibody.

The major advantage of these thiophilic gels over protein A gels is that they bind **all** antibodies with sufficiently high capacity and very little discrimination between subclasses or species. Furthermore, elution conditions are much milder, which might be beneficial if the harsh elution conditions required for strongly binding antibodies to protein A lead to their denaturation. Thiophilic gels also purify chicken IgY ***(4)***. A general method is provided in **Subheading 3.4**.

The major disadvantage of thiophilic gels is the requirement to add high concentrations of chaotropic salts to the adsorption buffer to facilitate binding. This requirement is not a problem for small-scale purifications at the bench, but it is unattractive at larger scale.

Recently, a major improvement of thiophilic chromatography came about with the utilization of very high ligand concentrations ***(9)***. By immobilizing mercaptoheterocyclics such as 4-mercaptoethyl pyridine at very high concentrations, the need to add chaotropic salts such as sodium sulfate was abolished. All antibodies investigated are adsorbed at neutral pH, independent of the salt concentration. At acidic pH values, the ligand becomes charged and through charge–charge repulsion, the antibody is eluted. This variation of thiophilic adsorption chromatography is highly reminiscent of protein A chromatography in its simplicity, but with the added advantage that it is nonspecific for antibodies. Purities and recoveries obtained range from 90% for antibodies from protein-free cell culture supernatant to approx 75% from ascitic fluid. The method is outlined in **Subheading 3.5**.

1.3. Immobilized Metal Affinity Chromatography

Immobilized metal affinity chromatography (IMAC) is a general term for a variety of different immobilization chemistries and metals utilized ***(10)***. The most commonly used gel for IMAC is nickel-loaded iminodiacetic acid (Ni-IDA) gel, used, for example, in kits for the separation of His-tail modified proteins. Under slightly alkaline conditions, the interaction between the immobilized nickel and proteins is strongest with accessible histidine residues. Ni-IDA binds to the Fc portion of the antibody ***(11)***, and similar to thiophilic adsorption chromatography, it binds all antibodies without discrimination between subclasses or species ***(11,12)***. Depending on the growth medium used, some contamination is apparent, namely transferrin and traces of albumin. The purity of monoclonal antibodies purified out of ascitic fluid is generally lower than out of hybridoma cell culture supernatant. As described for protein A chromatography, a shift in pH to acidic values leads to the generation of charges on the histidine residues and consequent elution of the protein bound. Alternatively, competitive elution with either imidazole or EDTA yields antibody in good purity. However, the elution with EDTA delivers the antibody with the metal still bound to the protein, while imidazole adsorbs at 280 nm, interfering with UV detection. A general protocol is outlined.

2. Materials

2.1. General Purification of Human IgG, Humanized IgG, and Mouse IgG2a and Mouse IgG2b Using Protein A

1. Buffer A: 50 m*M* Tris-HCl, pH 7.5.
2. Buffer B: 50 m*M* Tris-HCl, 500 m*M* NaCl, pH 8.0.
3. Buffer C: 100 m*M* acetate buffer, 50 m*M* NaCl, pH 3.0.
4. Buffer D: 10 m*M* NaOH.

2.2. General Purification of Mouse IgG1, Rat IgG1, and Rat IgG2b Using Protein A

1. Buffer A: 500 m*M* sodium citrate, ph 8–8.4.
2. Buffer B: 100 m*M* sodium acetate, 50 m*M* NaCl, pH 4.0.
3. Buffer C: 10 m*M* NaOH.

2.3. General Purification of IgG with Removal of Bovine IgG Using Protein A

1. Buffer A: 50 m*M* Tris-HCl, pH 7.5.
2. Buffer B: 50 m*M* citrate, pH 5.0.
3. Buffer C: 50 m*M* citrate, 50 m*M* NaCl, pH 3.0.
4. Buffer D: 10 m*M* NaOH.

2.4. Thiophilic Purification of IgG

1. Buffer A: 50 m*M* Tris-HCl, 500 m*M* sodium sulfate, pH 7.5.
2. Buffer B: 50 m*M* sodium acetate, pH 5.0.
3. Buffer C: 10 m*M* NaOH.

2.5. General Purification of IgG Using MEP-HyperCell

1. Buffer A: 50 m*M* Tris-HCl, pH 7.5.
2. Buffer B: 100 m*M* acetate buffer, 50 m*M* NaCl, pH 3.5.
3. Buffer D: 10 m*M* NaOH.

2.6. Immobilized Metal Affinity Purification of IgG

1. Buffer A: 50 m*M* sodium phosphate, 500 m*M* sodium chloride, pH 8.0.
2. Buffer B: 50 m*M* sodium acetate, 50 m*M* sodium chloride, pH 4.5.
3. Buffer C: 200 m*M* sodium phosphate, 200 m*M* sodium chloride, pH 8.0.

4. Buffer D: 50 m*M* Tris-HCl, 500 m*M* sodium chloride, 50 m*M* EDTA, pH 8.0.
5. Buffer E: Buffer B, 50 m*M* nickel chloride.

3. Method

3.1. General Purification of Human IgG, Humanized IgG, and Mouse IgG2a and Mouse IgG2b Using Protein A (*see* Note 1)

This protocol is designed for the purification of high-affinity IgG monoclonal antibodies from hybridoma cell culture supernatant and ascitic fluid. For lower affinity monoclonals, refer to **Subheading 3.2**. or the other purification methods described below.

For cell culture supernatants that contain fetal calf serum and the separation of contaminating bovine IgG from the monoclonal antibody, please see **Subheading 3.3**.

1. Bring all materials to room temperature.
2. Equilibrate a 10-mL protein A gel with 5 column volumes of buffer A.
3. Load supernatant onto protein A–gel column at 5 mL/min.
4. Load up to 20 mg of antibody/mL of gel.
5. Wash with 10 column volumes of buffer B.
6. Elute antibody with 5 column volumes of buffer C.
7. Reequilibrate column with 2 column volumes of buffer A.
8. Wash with 5 column volumes of buffer E followed by 10 column volumes of buffer A.

The column is ready for the next chromatography run.

3.2. General Purification of Mouse IgG1, Rat IgG1, and Rat IgG2b Using Protein A (*see* Note 2)

For cell culture supernatants that contain fetal calf serum and the separation of contaminating bovine IgG from the monoclonal antibody, please *see* **Subheading 3.3**.

1. Bring all materials to room temperature.
2. Dilute supernatant 1:1 with 1000 m*M* sodium citrate solution. Filter the resulting solution through a 0.2-µm filter **(important)**.
3. Equilibrate a 10-mL protein A gel with 5 column volumes of buffer A.
4. Load supernatant onto the protein A–gel column at 5 mL/min.
5. Load up to 12 mg of antibody/mL of gel.
6. Wash with buffer A until the UV baseline is reached.
7. Elute antibody with 10 column volumes of buffer B.
8. Reequilibrate column with 2 column volumes of buffer A.
9. Wash with 5 column volumes of buffer C followed by 10 column volumes of buffer A.

The column is ready for the next chromatography run.

3.3. General Purification of IgG with Removal of Bovine IgG Using Protein A (*see* Note 3)

This method is designed for the purification of human, humanized, and mouse IgG2a and IgG2b monoclonal antibodies from hybridoma cell culture supernatant containing fetal calf serum. It does not work well with weakly binding antibodies such as mouse IgG1.

1. Bring all materials to room temperature.
2. Equilibrate a 10-mL protein A gel with 5 column volumes of buffer A.
3. Load supernatant onto the protein A–gel column at 5 mL/min.
4. Load up to 20 mg of antibody/mL of gel.
5. Wash with 5 column volumes of buffer A.
6. Wash with 15 column volumes of buffer B.
7. Elute antibody with 10 column volumes of buffer C.
8. Wash with 5 column volumes of buffer E followed by 10 column volumes of buffer A.

The column is ready for the next chromatography run.

3.4. Thiophilic Purification of IgG (*see* Note 4)

This protocol is designed for the purification of all IgG from hybridoma cell culture supernatant and ascitic fluids. For cell culture supernatants that contain more than 5% fetal calf serum, the monoclonal antibody will be contaminated with bovine IgG.

1. Bring all materials to room temperature.
2. Dilute supernatant 1:1 with 1000 m*M* sodium sulfate solution.
3. Filter the resulting solution through a 0.2-μm filter (**important**).
4. Equilibrate thiophilic gel with 5 column volumes of buffer A.
5. Load supernatant onto the thiophilic gel column at 5 mL/min.
6. Load up to 10 mg of antibody/mL of gel.
7. Wash with buffer A until the UV baseline is reached.
8. Elute antibody with 10 column volumes of buffer B.
9. Wash with 5 column volumes of buffer C followed by 10 column volumes of buffer A.

The column is ready for the next chromatography run.

3.5. General Purification of IgG Using MEP-HyperCell (*see* Note 5)

This protocol is designed for the purification of all IgG from hybridoma cell culture supernatant and ascitic fluids. For cell culture supernatants that contain more than 5% fetal calf serum, the purity is relatively low at approx 50–65%.

1. Bring all materials to room temperature.
2. Equilibrate a 10-mL MEP-HyperCell gel with 5 column volumes of buffer A.
3. Load supernatant onto the column at 5 mL/min.
4. Load up to 20 mg of antibody/mL of gel.
5. Wash with 10 column volumes of buffer A.
6. Elute antibody with 8 column volumes of buffer B.
7. Reequilibrate the column with 2 column volumes of buffer A.
8. Wash with 8 column volumes of buffer E followed by 10 column volumes of buffer A.

The column is ready for the next chromatography run.

3.6. Immobilized Metal Affinity Purification of IgG (*see* Note 6)

This protocol is designed for the purification of all IgG from hybridoma cell culture supernatant and ascitic fluids. For cell culture supernatants that contain more than 5% fetal calf serum, the monoclonal antibody will be contaminated with bovine IgG.

1. Bring all materials to room temperature.
2. Dilute three parts of supernatant with one part of buffer C.
3. Equilibrate a 10-mL IMAC gel with 5 column volumes of buffer B.
4. Load the column with buffer E until the column is completely colored.
5. Wash with 5 column volumes of buffer B.
6. Equilibrate with 5 column volumes of buffer A.
7. Load supernatant onto the IMAC gel column at 5 mL/min.
8. Load up to 10 mg of antibody/mL of gel.
9. Wash with buffer A for 15 column volumes until the UV baseline is reached.
10. Elute antibody with 10 column volumes of buffer B.
11. Strip column with 5 column volumes of buffer D, followed by 5 column volumes of buffer B.

The column is ready for the next chromatography run.

3.7. Concluding Remarks

All the protocols provided in the yield antibodies in good yield and good purity. If not further specified in the accompanying notes, the protocols are usable with virtually all commercially available gels. Difficulties in using a protein A gel can be overcome by using a different affinity column such as a thiophilic gel or an IMAC gel. In most protocols provided, elution is accomplished at acidic pH values. Therefore, further purification can be accomplished by adsorbing the eluate onto a cation-exchange gel. Most of the impurities will flow through and, when using gradient elution, the antibody is generally the first protein to elute from the column.

4. Notes

1. This general protocol works well with high-affinity antibodies and is independent of the particular protein A gel used. The purity of the antibody recovered can be increased by first dialyzing the supernatant or the ascitic fluid against buffer A. Sometimes, phenol red is difficult to remove and the washing step has to be increased accordingly until no red color can be detected on the gel.
2. No pH adjustments have to be made for the sodium citrate solution as the pH will be between 8.0 and 8.5. It is very important to filter the resulting solution after mixing of the supernatant and the sodium citrate solution. In the case of ascitic fluid, a precipitate is clearly visible, while in the case of hybridoma cell culture supernatant precipitating material might not be visible. The purity is somewhat lower than in the case of high-affinity antibodies, but it is very acceptable in the 85–90% range.
3. The molarity of the citrate buffer used in the intermediate wash is slightly dependent on the protein A gel used. It is possible to wash the protein A gel from Pharmacia with 100 m*M* citrate buffer, pH 5.0, while the higher molarity would start to elute antibody from a protein A gel from BioSepra. A good starting point is the 50 m*M* citrate buffer outlined above, and if no loss of the desired monoclonal antibody is detected in the wash fraction, higher buffer concentrations can be employed.
4. There are only a few thiophilic gels commercially available. Of these, the thiophilic gel from E. Merck is by far the best. The most important precaution in using thiophilic gels is the need to filter the supernatant after adjustment to 500 m*M* sodium sulfate. Other antibody molecules like IgA ***(5)***, bispecific antibodies ***(6)***, and single-chain antibody fragments have been purified using thiophilic gels ***(7,8)***.
5. All antibodies, independent of species and subclass, can be purified with this gel. No separation of subclasses can be achieved, however. It is highly likely that similarly to the thiophilic gels described above, other antibody classes such as IgA, IgE, IgM, and chicken IgY can be purified using MEP-HyperCell. The MEP-HyperCell resin is available from BioSepra Inc., a unit of Ciphergen, Inc.
6. Generally, all chelating and amine-containing reagents such as citrate and Tris buffers should be avoided in the feedstocks and buffers used, as these buffers will strip the column of the immobilized metal. If possible, using a gradient to elute the proteins results in better purity of the antibody sought. Three different metals can be used to purify antibody, namely copper, nickel, and cobalt. A good starting metal of any purification using IMAC is nickel. Other metals that work in the purification of antibodies are cobalt and copper. The use of zinc does not yield any antibody. The addition of a

His-tag allows the specific adsorption and elution of single-chain antibodies and antibody fragments ***(13,14)***.

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A Rapid Method for Generating Large Numbers of High-Affinity Monoclonal Antibodies from a Single Mouse

Nguyen Thi Man and Glenn E. Morris

1. Introduction

Since the first description by Kohler and Milstein ***(1)***, many variations on this method for the production of monoclonal antibodies (MAb) have appeared and recent comprehensive books on the subject have included both recombinant and conventional methods (*see* ***Further Reading***). The variation we describe here, however, includes a number of refinements that enable rapid (6–10 wk) production from a single spleen of 10–30 cloned and established hybridoma lines producing antibodies of high-affinity. We initially applied this method to recombinant fusion proteins containing fragments of the muscular dystrophy protein dystrophin ***(2–5)***, and dystrophin-related proteins ***(6,7)***, to hepatitis B surface antigen ***(8)*** and to the enzyme creatine kinase (CK) ***(9)***, and have used the MAb thus produced for immunodiagnosis, epitope mapping, and studies of protein structure and function ***(5–12)***. Epitopes shared with other proteins are common, so availability of several MAb against different epitopes on a protein can be important in ensuring the desired specificity in immunolocalization and Western blotting studies ***(6)***. More recently, we have applied this same technique for nuclear proteins, such as emerin (16 MAbs; ***13***), lamin A (5 MAbs; ***14***), SMN (22 MAbs; ***15***), gemins (35 MAbs; ***16***) and the transcription factor, Six5 (18 MAbs; ***17***), as well as other human proteins as diverse as huntingtin (19 MAbs; ***18***) and dopamine receptors (8 MAbs; ***19***).

In the standard Kohler and Milstein method, Balb/c mice are immunized with the antigen over a period of 2–3 mo, and spleen cells are then fused with mouse myelomas using polyethylene glycol (PEG) to immortalize the B-lymphocytes secreting specific antibodies. The hybrid cells, or hybridomas, are then selected

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using medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) ***(20)***. All unfused myelomas are killed by HAT medium, and unfused spleen cells gradually disappear in culture; only hybridomas survive.

Use of hyperimmune mice is critical for success and we have found this much easier to achieve using recombinant proteins as immunogens, rather than conjugated peptides. Fusion proteins with a bacterial component, such as pET vectors with a thioredoxin fusion (Novagen, Inc), have been especially useful. We have rarely found it necessary to take steps to ensure correct folding of the protein immunogen, since some MAbs that recognize both folded and unfolded protein were usually produced when appropriately screened. An exception that did require special steps in both immunogen preparation and hybridoma screening to obtain MAbs against native protein is described below. Fig.1A illustrates both a weak response to immunogen (little binding at 1:1000 dilution), making MAb production difficult, and a typical strong response (strong binding at 1:1000 dilution) that will make hybridoma production very much easier. The importance of appropriate screening of both mouse test bleeds and hybridoma supernatants cannot be emphasized too often. If the desired antibody is not represented in the mouse serum, there is little chance of its emerging after hybridoma fusions.

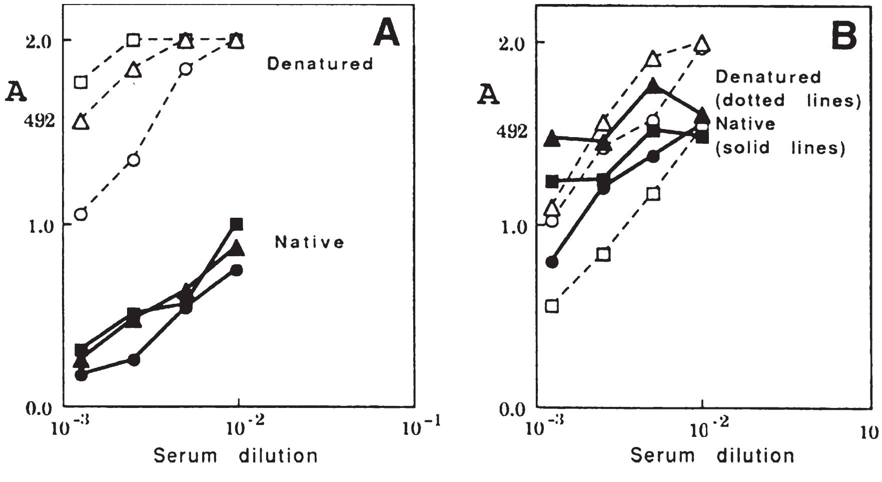

Fig. 1. Effect of aggregation of CK on the immune response in Balb/c mice. Test sera were taken from three mice immunized with untreated CK (**A**): circles, squares, and triangles represent individual mice) or with glutaraldehyde-aggregated CK (**B**), and serial dilutions were used as primary antibody in either direct ELISA for denatured CK (dotted lines) or sandwich ELISA for native CK (solid lines). (Reproduced with permission from **ref. *9***.)

Special features of the detailed method to be described here include:

1. A culture medium for rapid hybridoma growth without feeder layers;
2. Screening early to select high-affinity antibodies;
3. Cloning without delay to encourage hybridoma growth and survival; and
4. The use of round-bottomed microwell plates to enable rapid monitoring of colony growth with the naked eye.

In typical fusion experiments, cells are distributed over ten microwell plates (960 wells) and 200–700 wells show colony growth. Of these hybridomas, up to 50% (100–300) produce antibodies that show some antigen binding, though many may be too weak to be useful. After further screening for desired affinity and specificities, we normally select 25–30 as a convenient number to clone. **Table 1** illustrates the results obtained from four fusions by this method. Only occasionally, in our experience, are two or more MAb produced that are indistinguishable from each other when their epitope specificities are later examined in detail.

In the early stages after fusion, a particularly good cell-culture system is needed to promote rapid hybridoma growth in HAT medium. Horse serum, selected for high cloning efficiency, is a much better growth promoter than most batches of fetal calf serum, and HAT medium is prepared using myeloma-conditioned medium and human endothelial culture supernatant, a hybridoma growth promoter. Under these conditions, colonies are visible with the naked eye 7–10 d after fusion, and screening should start immediately. Even unimmunized mouse spleens will produce large numbers of colonies, so the use of hyperimmunized mice, with high serum titers in the screening assay, is essential for generating a high proportion of positive colonies. The screening assays are also of critical importance, because they determine the characteristics of the MAb produced. Both these points are illustrated in **Fig. 1**. CK, a fairly typical

Table 1
Examples of Colony and MAb Yields Using This Method

Antigen	Wells with growth	Wells binding to antigen strongly	Wells with desired affinity//specificity	Final no. of MAb
108–kDa rod fragment of dystrophin in fusion protein ***(2)***	587	136	27	16
59–kDa rod fragment of dystrophin in fusion protein ***(4)***	669	147	30	25
55–kDa C--terminal fragment of dystrophin in fusion protein ***(5)***	700+	42	20	17

"globular" enzyme, is denatured and inactivated when attached directly to ELISA plastic, but it can be captured onto ELISA plates in its native form by using Ig from a polyclonal antiserum *(9)*. When mice were immunized with untreated CK, antibody titers against denatured CK were high, whereas titers of antibody against native CK were insufficient to produce a plateau in the capture ELISA, even at high serum concentrations (**Fig. 1A**). Seven fusions from such mice (over 1000 colonies screened) produced only two, low-affinity MAb specific for native CK. In contrast, when we immunized with CK aggregated by glutaraldehyde treatment (**Fig. 1B**), titers were higher for native CK antibodies than for those against denatured CK. In a fusion performed with one of these mice, only 2% of the fusion wells were positive in the direct ELISA (denatured CK), but 13% recognized only native CK in the capture ELISA *(9)*. This illustrates the importance of having both high-titer mice and a suitable screen.

Finally, although the method is rapid and efficient, it is also labor-intensive, especially during screening and cloning. When embarking on the method, one should not be daunted by the prospect of having over 70 culture plates in the incubator simultaneously by the time that the later cloning stages of a single experiment are reached.

2. Materials

1. One or two vertical laminar flow sterile hoods (e.g., Machaire, Bolton, UK).
2. 37°C CO_2 incubator (e.g., LEEC, Nottingham, UK; *see* **Note 1**).
3. Transtar 96 (Corning Inc, Lowell, MA) for plating fusions and removing hybridoma supernatants.
4. Round-bottomed 96-well tissue-culture microwell plates (Invitrogen, Carlsbad, CA, cat. no. 163320) (*see* **Note 2**).
5. Sterile tissue-culture flasks 25 mL (Invitrogen), 75 mL (Invitrogen), and 24-well plates (Invitrogen), 1.8-mL cryogenic vials (AlphaLabs, Eastleigh, UK), and sterile 25-mL universal containers (Invitrogen).
6. Freund's adjuvant, complete (Sigma [St. Louis, MO] no. F-5881) stored at 4°C.
7. Freund's adjuvant, incomplete (Sigma) stored at 4°C.
8. Pristane (2,6,10,14 tetramethyl pentadecane; (Sigma) stored at room temperature.
9. 500X HT: 6.8 mg/mL hypoxanthine (Sigma) and 1.95 mg/mL thymidine (Sigma) in water (*see* **Note 3**). Filter to sterilize. Store in 5-mL aliquots in sterile plastic universals at −20°C (stable for a few years).
10. 1000X Aminopterin (Sigma): 4.4 mg are dissolved in 25 mL of water (*see* **Note 3**). Since the chemical is toxic and teratogenic, it must be weighed with appropriate containment and operator protection. Filter to sterilize. Store in 2-mL aliquots at −20°C, wrapped in aluminum foil to protect from light (stable for years).
11. 50% polyethylene glycol (PEG), mol wt 1500 (Merck, Darmstadt, Germany), is made up by weighing 10 g of PEG in a glass universal and autoclaving. Although the solution is still hot (about 60°C), add 10 mL of DMEM/25 m*M* HEPES

prewarmed in a 37°C water bath. Mix well, and store in 5-mL aliquots at room temperature in the dark; stable at least for 6 mo. The pH should be slightly alkaline (as judged by phenol red) when used for fusion.

12. DMEM/Glutamax I/25 m*M* HEPES. (Invitrogen). This is used as a "physiological saline," not for growing cells.
13. DMEM/Glutamax I/20% HS is prepared by adding to each 80 mL of 1X DMEM-Glutamax I (Invitrogen): 1 mL penicillin-streptomycin (Invitrogen, cat. no. 15070-063), 1 mL nonessential amino acids (MEM) (Invitrogen, cat. no. 11140), and 20 mL selected horse serum (HS). For routine growth and maintenance of both myelomas and established hybridomas, serum is reduced to 10%.
14. Cloning medium: DMEM/20% HS supplemented with 1X HT and 5% human endothelial culture supernatant (HECS) (No longer available from Costar-Corning; *see* **Note 16**).
15. HAT medium: Myeloma-conditioned DMEM-Glutamax I/20% HS with 1X HAT and 5% HECS.
16. Medium for feeding the fusion: DMEM-Glutamax I/20% HS with 5% HECS and 5X HT.
17. Medium for freezing down cells: 90% HS/10% dimethyl sulfoxide (DMSO) (Sigma).
18. Trypan blue (0.1%) (Sigma) in PBS.

3. Methods

3.1. Immunization of Balb/c Mice

1. Purify the protein antigen. With purified antigen, screening is faster and easier, and the yield of MAb is higher, but proteins of 50% purity or less on SDS-PAGE may still give good results.
2. Each of three Balb/c mice (6–8 wk old) is given an ip injection of 50–100 µg of antigen in 0.1 mL PBS emulsified with an equal volume of Freund's complete adjuvant by sucking up and down many times into a disposable sterile plastic 2-mL syringe with a sterile 21-gage 1–1/2 in. no. 2 needle (*see* **Note 4**).
3. Four weeks later, an ip boost of 50–100 µg antigen in Freund's incomplete adjuvant is given.
4. Ten days later, blood (c. 0.1 mL) is taken from the tail vein, allowed to clot, and centrifuged at 10,000 rpm for 5 min. The serum is tested by the method to be used for screening hybridomas. If the titers are high in this assay (*see* **Fig. 1**), the mice should be left to rest for an additional month before fusion.
5. The best mouse is boosted with 50–100 µg of antigen in PBS, ip, and/or iv in the tail vein, 4, 3, and 2 d before fusion.

3.2. Selection of Horse Serum

1. Most suppliers will provide test samples of different serum batches and keep larger amounts on reserve. All sera are heat-inactivated at 56°C for 30 min before use, though this is not known to be essential. Thaw serum in 37°C water bath and swirl to mix before incubating in a 56°C water bath for 30 min with occasional swirling.

We have never yet found a batch of fetal bovine serum that performs as well as horse serum in these tests (*see also* **Subheading 3.8**).

2. Prepare cells of any hybridoma or myeloma line at 1000 cells/mL in DMEM and dilute to 40 cells/mL, 20 cells/mL, 10 cells/mL in DMEM/20% HS of different batches (cloning of established cell lines does not require feeder layers or HT and HECS). For each test serum, plate one 96-well round-bottomed microplate with four rows at four cells in 0.1 mL/well, four rows at two cells in 0.1 mL/well, and four rows at one cell in 0.1 mL/well.
3. From the fifth day onward, note the number of colonies at each cell density for each test serum, as well as the size of the clones. After 2 wk, choose the batch of serum with the highest number and the largest clones. Serum may be stored at −70°C for at least 1–2 yr without loss of activity.

3.3. Growth and Maintenance of Myelomas

SP2/O ***(21)*** myeloma lines grow very fast and do not synthesize immunoglobulins They should be kept in logarithmic growth in DMEM/5% HS for at least a wk before fusion by diluting them to 5×10^4/mL when the cell density reaches 4 or 5×10^5/mL. If thawing myelomas from liquid nitrogen, it is advisable to begin at least 1–2 wk before fusion (*see* **Note 5**). We have never found it necessary to treat lines with azaguanine to maintain their aminopterin sensitivity, which can be checked by plating in HAT medium at 100 cells/well.

3.4. Fusion with Spleen Cells

1. All dissection instruments are sterilized by dry heat (160°C for 5 h), autoclaving (120°C for 30 min) or dipping in 70% ethanol, and flaming in a Bunsen burner. Two separate fusions are done with each spleen as a precaution against accidents.
2. Two days before fusion, set up 2 × 75-mL flasks with 40 mL each DMEM 20% HS and 1×10^5 myeloma cells/mL (Sp2/O).
3. On the day of fusion, place in a 37°C water bath 100 mL of DMEM/25 m*M* HEPES, 10 mL of DMEM/20% HS, and 1 bottle of PEG1500.
4. Count the myeloma cells in a hemocytometer after mixing an aliquot with an equal volume of 0.1% Trypan blue in PBS; cell density should be $4–6 \times 10^5$ with viability 100% and no evidence of contamination.
5. Transfer the myeloma cells to four universals and centrifuge for 7 min at 300 *g* (MSE 4 L). Remove the supernatants with a 10-mL pipet and retain them for plating the fusion later (myeloma-conditioned medium). Resuspend each pellet in 5 mL of DMEM/HEPES, and combine in two universals (2 × 10 mL).
6. The immunized mouse is killed by cervical dislocation outside the culture room and completely immersed in 70% ethanol (15–30 s). Subsequent procedures are performed in sterile hoods. The mouse is pinned onto a "sterile" surface (polystyrene covered with aluminum foil and swabbed with 70% ethanol), and a small incision in the abdominal skin is made with scissors. The peritoneum is then exposed by tearing and washed with 70% ethanol before opening it and pinning it

aside. The spleen is lifted out, removing the attached pancreas with scissors, and placed in a sterile Petri dish (*see* **Note 6**).

7. After removing as much surrounding tissue as possible, the spleen is placed in 10 mL of DMEM/25 m*M* HEPES and held at one end with forceps while making deep longitudinal cuts with a sterile, curved scalpel to release the lymphocytes and red blood cells. This process is completed by scraping with a Pasteur pipet sealed at the end until only connective tissue is left (2–3 min).
8. The spleen cell suspension is transferred to a 25-mL universal, with pipeting up and down five to six times to complete the dispersal. Large lumps are allowed to settle for 1 min before transferring the supernatant to a second universal and centrifuging for 7 min at 300 *g*. Resuspend the pellet in 5 mL of DMEM/25 m*M* HEPES, and keep at room temperature. Usually about 1 × 10^8 cells/spleen are obtained (*see* **Note 7**).
9. Add 2.5 mL spleen cell suspension to each 10 mL of myeloma cell suspension, and mix gently. Centrifuge at 300 *g* for 7 min at 20°C. Remove supernatants with a pipet, and resuspend pellets in 10 mL DMEM/25 m*M* HEPES using a pipet. Centrifuge at 300 *g* for 7 min. Remove the supernatant completely from one pellet (use a Pasteur pipet for the last traces), and then loosen the pellet by tapping the universal gently (*see* **Note 8**).
10. Remove the DMEM/25 m*M* HEPES and the 50% PEG from the water bath just before use. Take 1 mL of PEG in a pipet, and add dropwise to the cell pellet over a period of 1 min, mixing between each drop by shaking gently in the hand. Continue to shake gently for another minute. Add 10 mL DMEM/25 m*M* HEPES dropwise with gentle mixing, 1 mL during the first minute, 2 mL during the second, and 3.5 mL during the third and fourth minutes. Centrifuge at 300 *g* for 7 min. (This procedure can be repeated with the second cell pellet, while the first is spinning.)
11. Remove supernatant, and resuspend pellet gently in 5 mL of DMEM/20% HS. Place both 5-mL cell suspensions in their universals in the CO_2 incubator for 1–3 h.
12. During this time, take the c. 80 mL of myeloma-conditioned medium and add 4.5 mL of HECS, 90 µL of aminopterin, and 180 µL of hypoxanthine/thymidine solutions. Filter 2 × 40 mL through 0.22-µm filters (47 mm; Millipore [Bedford, MA]) to resterilize, and remove any remaining myeloma cells.
13. To each 40 mL, add the 5-mL fusion mixture from the CO_2 incubator and distribute in 96-well microtiter plates (4 plates/fusion; 100 µL/well) using a Transtar 96 or a plugged Pasteur pipet (3 drops/well) (*see* **Note 9**). Put the plates from each fusion in a separate lunch box in the CO_2 incubator and leave for 3 d.
14. On d 4, add to each well 80 µL of DMEM/20% HS supplemented with HECS and 5X HT. Replace in the CO_2 incubator as quickly as possible.
15. By d 10–14, a high proportion of the wells should have a clear, white, central colony of cells, easily visible with the naked eye. If you want to select for high-affinity MAb (and you would be well advised to do so, unless you have some very special objectives), do not delay screening. If you are using a sensitive screening method, such as ELISA, immunofluorescence, or Western blotting, you will detect

high-affinity antibodies from even the very small colonies. Do not wait for the medium to turn yellow, or your cells may start to die (if you are using a less sensitive screen, such as an inhibition assay, you *may* need to wait longer). A high proportion of the 768 wells (50–100%) should have colony growth by 14 d. If it is <10%, try reducing the PEG concentration. If you regularly obtain over 70%, use 6–8 plates/fusion instead of 4. There should be no microbial contamination (*see* **Note 10**).

3.5. Screening

It would be quite wrong to describe one screening method in detail, since this must be chosen to suit the purpose for which MAb are required. We recommend ELISA as the simplest technique for screening 8 × 96 wells in 1 d, but for coating ELISA plates directly, 10–50 µg of reasonably pure antigen are required. Alternatively, plates can be coated with a capture antibody; we have used this approach to capture CK in its native conformation ***(9)*** and to capture hepatitis B surface antigen from human plasma ***(8)***. The availability of multichannel miniblotters makes direct screening by Western blotting feasible, and with two miniblotters, 224 microwells can be screened in one long day. Immunohistochemistry on cultured cells grown in microwells is also feasible, though we have not used it ourselves. A Transtar 96 apparatus is a rapid and sterile way to remove 10–50 µL of culture supernatant from all 96 wells of a microwell plate. The culture plates have to be open inside the hood for only a few seconds. They should always be wiped with paper tissue soaked in 70% ethanol before opening. Screening the whole plate, including wells without cell growth, by ELISA avoids possible errors when trying to keep track of individual wells. If ELISA-positive wells are to be screened further (e.g., by Western blotting using a multichannel miniblotter), this first 50 µL of supernatant may be diluted into 100 µL or more of PBS; multiple sampling of fusion wells should be minimized to avoid contamination. We usually screen by ELISA on d 10, carry out further screening of ELISA-positive wells on d 11 and 12, and clone about 30 of the best wells on d 12, 13, and 14.

3.6. Cloning by Limiting Dilution

1. Using a plugged Pasteur pipet, transfer the positive clone to a 24-well plate containing 0.5 mL of cloning medium (*see* **Note 11**).
2. Take an aliquot of these cells, dilute with an equal volume of 0.1% trypan blue in PBS, and count cells on at least two chambers of a hemocytometer.
3. Prepare 6 mL of 160 cells/mL and perform serial dilutions to 40 cells/mL and 10 cells/mL. Plate four rows at 16 cells in 0.1 mL/well, four rows at four cells in 0.1 mL/well, and four rows at one cell in 0.1 mL/well. Three drops from a plugged Pasteur pipet are about 0.1 mL.
4. Eight to ten days after plating, clones at 1 cell/well are visible, and screening can start immediately. It is best to screen at least 16–24 wells from each cloning plate.

As soon as positive clones have been identified, they should be cloned a second time, but at 4, 1, and 0.5 cells/well instead of 16, 4, and 1.

5. When these plates are screened again after another 8–10 d, at least one dilution should have <50% of the wells with growth, and *all wells with growth* should be positive in the screening. If any colonies are negative, cloning should be repeated until all wells are positive (*see* **Note 12**).

3.7. Preservation of Hybridoma Lines

1. Hybridoma clones should be expanded slowly from microwells, first into 0.5 mL in 24-well plates, feeding to 1 or 2 mL before transferring to a 25-mL flask in 5 mL of medium.
2. To preserve cell lines indefinitely, centrifuge 1–3 × 10^6 growing cells in a universal, remove all supernatant, suspend cell pellet in 0.3–0.5 mL of ice-cold 7.5% DMSO/HS, and transfer the suspension into a 1.8-mL cryotube.
3. Surround the cryotube with 1 in of polystyrene, and place in a −70°C freezer. Transfer it the next day to a liquid nitrogen container.
4. It is advisable to freeze several vials of the same line and thaw one after a week to ensure viability, while maintaining the cells in culture.
5. To recover cells from liquid nitrogen, thaw the cryovial quickly in a 37°C water bath until only a tiny piece of ice is visible. Transfer the cell suspension immediately to 10 mL of ice-cold DMEM/20% HS. Centrifuge at 300 *g* for 7 min, remove supernatant completely, and resuspend the cell pellet in 3–5 mL of cloning medium. Transfer the cell suspension into a 25-mL flask, reserving about 0.1 mL to count viable cells, and place flask in the CO_2 incubator. If the viable cell count is <1 × 10^4/mL, cloning by limiting dilution should be carried out immediately (*see* **Note 13**).

3.8. Antibody Production

1. Antibody can be generated as culture supernatant by starting a flask culture at 1 × 10^5/mL in DMEM/20% HS and, when the cell density reaches 4–6 × 10^5/mL, diluting to 1 × 10^5/mL. The concentration of horse serum is gradually reduced to 10% in the process of reaching the volume required (*see* **Note 14**). The cells are then left to grow undisturbed until they are all dead (*see* **Note 15**). Culture supernatant can then be harvested by centrifugation, and stored in aliquots at −20°C until required. For routine use, keep an aliquot of 1–2 mL with 0.1% sodium azide at 4°C. Antibody prepared in this way contains large amount of horse Ig which co-purifies with mouse Ig and is undesirable if direct labelling of the purified MAb is planned. Hybridoma cell lines can be switched to growth media containing fetal bovine serum (which has low endogenous Ig levels) or to various proprietary serum-free or protein-free media designed for hybridomas. In our experience, however, MAb levels are at least 10X lower than can be achieved with horse serum. Ascites production is an easy way to produce milligram quantities of MAb of sufficient purity for labelling, but this process is discouraged by regulatory authorities in many countries because of the distress caused to mice. Ascites production is available commercially, but a method is also given here.

2. For ascites fluid, an adult Balb/c mouse is primed with 0.2 mL of pristane ip.
3. After 7–10 d, 1–3 × 10^6 hybridoma cells in logarithmic growth (100% viability) are centrifuged at 300 *g* for 7 min at 20°C and washed once with DMEM/25 m*M* HEPES to remove all serum. The cell pellet is resuspended in 0.2 mL of DMEM/HEPES, and injected ip into a primed mouse using a 21-gage no. 2 needle (green).
4. Ascites development becomes evident by external examination within 7–14 d and mice must be examined twice a day over this period. Mice are killed by cervical dislocation as soon as they show signs of discomfort (advice should be sought and followed). The peritoneum is exposed as in **Subheading 3.4**, **step 6**, and a 21-gage needle on a 5-mL syringe is inserted about 1 cm so that the tip remains visible through the peritoneum and fluid can be withdrawn without blockage. Last traces of fluid are removed after opening the cavity with scissors. The final volume obtained is usually 2–3 mL, though blood from the heart and thoracic cavity can also be collected (about 0.5 mL) and processed separately as a source of antibody.
5. Fluids are allowed to clot and then centrifuged at 3000 *g* for 20 min. Store in aliquots of 200 µL at −20°C or preferably −70°C.

4. Notes

1. Fusions from a single mouse spleen aimed at producing 20–30 cloned hybridoma lines by the method described here can eventually fill a standard size bench-top incubator completely. The floor of the incubator is filled with autoclaved, double-distilled water. The interior of the incubator must be kept as free of contamination as possible; this may necessitate removing the contents, changing the water, and wiping the interior with 70% ethanol on several occasions during hybridoma growth and cloning. Even so, we use large plastic lunch boxes to insulate unsealed culture vessels (e.g., microwell plates) from the turbulent incubator environment (a fan is desirable for uniform temperature and humidity). Holes plugged with cotton wool at each end of the lunch box to admit CO_2 are optional; an alternative is to leave the lids ajar for 1–2 min to equilibrate before closing them. They can be wiped regularly, inside and out with 70% ethanol. Never use toxic chemicals (e.g., bleach) to decontaminate an incubator. Never handle culture plates without gloves.
2. Cells roll to the bottom giving a white colony in the exact center of each well, visible even at early stages; flat-bottomed plates have initially transparent colonies, often at the side of the well and easily overlooked. Use of round-bottomed plates makes it very easy to monitor colony growth after fusion without using a microscope.
3. The mixture does not dissolve by itself, so add 1 *M* NaOH dropwise while stirring until the solution goes clear.
4. Avoid syringes with rubber plunger tips; when the emulsion thickens, they come off the plunger.

5. The myeloma cells should look uniform and healthy by this time with no sign of cell debris.
6. We normally perform all steps up to this point in a separate sterile hood. The mouse is nonsterile externally, and particular care is taken to avoid hairs; the outer skin is torn, rather than cut, for this reason. Mice should not be introduced into the hood used for cell culture.
7. Some protocols remove red blood cells by differential lysis at this point, but we have not found it necessary.
8. The final concentration of PEG during fusion is thought to be critical for the yield of colonies. If initial yields are low, try adding increasing (but small) amounts of DMEM/25 m*M* HEPES to the pellet before adding PEG.
9. Never use unplugged micropipet tips for adding to culture plates; sterile tips can be used for *removing* culture supernatant only.
10. Bacterial and yeast contaminations rarely occur and are usually the result of a major failure in sterile technique (e.g., inadequate sterilization of culture medium or glassware). Sources of any sporadic fungal contamination should be tracked down and eliminated. Some protocols recommend freezing down uncloned or partly cloned cells as a precautionary measure. Following our rapid cloning protocol, however, the first round of cloning and screening is often complete before the original colony is sufficiently expanded for freezing. It is certainly advisable to keep the original culture alive, however, by adding 0.1 mL of cloning medium back to the fusion well and by feeding the 24-well culture, if necessary. Cloning should not be regarded as an ordeal suitable only for healthy cells, but rather as a means of invigorating a failing culture. We always clone twice for this reason, even if the line is evidently clonal after the first round. *As a general maxim*: if in doubt or trouble, clone immediately.
11. There are rare exceptions to the general principle that cloning should be continued until all colonies are positive in the screens. We were once performing an initial screen by ELISA and then testing ELISA-positive wells by immunofluorescence microscopy (IMF) on muscle sections. After cloning one well that was positive in both assays, we found that only half the clones were ELISA-positive and very few of these were also IMF-positive. We recloned wells that were positive in both assays again with the same result. We thought we had come across our first "unstable" hybridoma, but by chance we tested ELISA-negative wells in IMF and found they were all IMF-positive. Only then did we realize that the original fusion well had contained two different hybridomas, one ELISA-positive and one IMF-positive. The purpose of cloning is to separate such lines, but by selecting wells that were positive in both assays (and hence still had two clones), we had been systematically defeating this objective.

12. When very few cells survive after being kept in liquid nitrogen, “cloning” by limiting dilution at about 100 total cells/well may be the only way to recover the cell line.
13. Cells may also be collected by centrifugation and resuspended in 5% fetal calf serum at this stage, if a culture supernatant with low levels of nonmouse Ig is required.
14. Most MAb are stable for long periods in sterile culture medium, but there are undoubtedly some that lose activity rapidly even at 4°C. These are perhaps best avoided, but, if required, bulk culture would have to be monitored regularly and supernatants harvested when their antibody activity is still high.
15. HECS (human endothelial culture supernatant) is no longer commercially available but can be prepared by growing HUVEC cells for 2 days to 80% confluence in Endothelial Cell Growth Medium (Promocell, Heidelberg, Germany, Cat No. C22020) and using the supernatant (store at –20°C).

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